Adipose Tissue Release of Interleukin-6 (IL-6) and Asymmetric Dimethyl Arginine (ADMA): Implications for Obesity Associated Metabolic Disease

Mohammad Javad Hosseinzadeh Attar MD
Department of Medicine
University College London

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Obesity is associated with the development of various metabolic diseases. Adipose tissue-derived factors may underlie this relationship. Two novel adipose signals associated with increased risk of coronary heart disease were investigated; interleukin-6 (IL-6) and the endogenous nitric oxide inhibitor, asymmetric dimethyl arginine (ADMA).

The effect of the cyclo-oxygenase (COX) pathway on basal adipose IL-6 production was examined. Basal COX-2 expression was detected in adipose tissue explants. There was a dose-dependent decrease in adipose IL-6 release by a non-selective COX inhibitor, aspirin. Cyclic AMP, and not Ca^{2+}, was the intracellular mediator of IL-6 release. PGE2 EP2 and 4 signalling is mediated by elevation in intracellular cAMP and agonists for these receptors elevated IL-6. Thus, basal IL-6 secretion occurs through increased COX-2 mediated PGE2 release signalling via EP4 receptors and elevated intracellular cAMP.

The role of the COX pathway was also investigated in adipogenesis. Aspirin and SC-560, a selective COX-1 inhibitor, inhibited adipocyte differentiation mainly by down-regulating adipogenic transcription factors. However, NS-398, a COX-2 selective inhibitor, was found to have no such effect. Thus, adipogenesis was found to be regulated by a COX-1 mediated mechanism.

ADMA, an endogenous NO inhibitor, is cleared mainly by catabolism by DDAH. Significant amounts of DDAH 1 and 2 mRNA and protein were expressed in mouse and human adipose tissue and adipocytes. In human subjects, the abdominal sub-cutaneous adipose tissue released ADMA *in vivo* and circulating levels in morbid obesity were elevated. Furthermore, weight loss increased adipose DDAH expression and decreased systemic ADMA

levels. *In vitro* studies also showed a direct correlation between the amount of adipose tissue and its release of ADMA. Thus, genetic, dietary and pharmacological disruption of DDAH altered adipose ADMA release.

In conclusion, this work showed that two important enzymes, COX and DDAH, in adipose tissue have the capacity to modulate cardiovascular risk in obesity by their regulation of IL-6 synthesis, adipogenesis and the release of ADMA.

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Declaration:

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Abbreviations

ACTH	adrenocorticotrophic hormone
ADA	American diabetes association
ADMA	asymmetric dimethyl arginine
AT	adipose tissue
ATP	adenosine triphosphate
BCS	bovine calf serum
BMI	body mass index
BSA	bovine serum albumin
C/EBP	CCAAT enhancer binding protein
Ca2+	calcium
cAMP	cyclic adenosine monophosphate
CCS	cosmic calf serum
cDNA	complementary DNA
cGMP	cyclic GMP
CHD	coronary heart disease
CO2	carbon dioxide
COX	cyclooxygenase
CRH	corticotrophin releasing hormone
CRP	C-reactive protein
CT	Computerized Tomography
CVD	cardiovascular disease
dbcAMP	dibutyryl cAMP
DDAH	dimethylarginine dimethylaminohydrolase
DEPC	diethylpyrocarbonate
DEX	dexamethasone
DEXA	Dual emission X-ray absorptiometry
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay

ER	endoplasmic reticulum
ETDRS	Early Treatment Diabetic Retinopathy Study
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gp130	glycoprotein-130
HCL	Hydrochloric acid
HDL	high-density lipoprotein
HOMA	homeostasis model assessment
HOT	Hypertension Optimal Treatment
HPA	Hypothalamic-Pituitary-adrenal
HPLC	high performance liquid chromatography
IBMX	Isobutylmethylxanthine
IFN-β	interferon β
IGF-1	insulin-like growth factor 1
IL-10	interleukin-10
IL-17	interleukin-17
IL-1β	interleukin-1β
IL-1ra	interleukin-1 receptor antagonist
IL-4	interleukin-4
IL-6	interleukin-6
IL-6R	interleukin-6 Receptor
IL-8	interleukin-8
IP3	inositol 1,4,5-triphosphate
IP3	inositol 1,4,5-triphosphate
KDA	kilo Dalton
KHZ	KiloHertz
LDL	low-density lipoprotein
L-NMMA	N(G)-monomethyl-L-arginine
LPL	lipoprotein lipase
LPS	lipopolysaccharide
MRI	magnetic resonance imaging
mRNA	messenger RNA
mV	miliVolt
N2	nitrogen
NAO	National Audit Office

NCX4016	NO-Aspirin
NEFA	non-esterified fatty acids
NF-kB	nuclear factor-kB
NIDDM	non-insulin dependent diabetes mellitus
NO	nitric oxide
NOS	nitric oxide synthase
NP-40	nonidet P-40
NS	not significant
NSAID	non-steroidal anti-inflammatory drug
OD	optical density.
PAI-1	Plasminogen activator inhibitor type 1
PBS	phosphate buffer saline
PBST	phosphate buffer saline plus Tween
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PG	prostaglandin
PGE2	prostaglandin E2
PPAR	peroxisome proliferator activated receptor
PRMT	protein arginine N-methyltransferase
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid.
RyR	ryanodine receptors
RyR	ryanodine receptors
SA	salicylic acid
SDS	sodium dodecyl sulphate
sgp130	soluble gp130
sIL-6 R	soluble interleukin-6 receptor
Sv	stromal vascular
TEMED	tetramethylethylenediamine
TNF sR-I	Tumor necrosis factor soluble receptor I
TNFα	tumour necrosis factor-α
TNFa sR-II	Tumor necrosis factor soluble receptor II
TZD	thiazolidinediones
Vit-E	vitamin E

VLDL	very low density lipoprotein
WAT	white adipose tissue

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Obesity

1.1.1 Epidemiology of obesity

Obesity is not a new phenomenon. It dates back to the Stone Age, this being detected through archaeological evidence. However, it has never been as common as it is today and across such a large proportion of the world. Currently, the prevalence of obesity is increasing globally (WHO, 1997). Over the past two years, a number of reports have repeatedly underlined the gravity of an impending worldwide public health crisis if current trends in weight gain and obesity are not tackled. Worldwide, one in 10 children and more than 1 billion adults are overweight and at least 300 million adults are clinically obese (Kimm et al, 2002). One prediction is that two thirds of European citizens will be overweight by the year 2030. Experts predict that the health consequences of this could be disastrous.

Obesity is a critical public health problem that causes millions of people to suffer unnecessary health problems and die prematurely. This is a condition that contributes to approximately 400,000 deaths each year, drains the economy of billions of pounds annually through direct and indirect medical expenses, disability and lost productivity (Allison et al, 1999; Katzmarzyk et al, 2004). The problem does not only affect developed countries. There is now a significant increase in overweight and obesity throughout the developing world. It affects all age ranges and both genders in many countries. It is estimated that more people will die from complications of overnutrition than of starvation (Rossner, 2001).

Obesity is defined in most instances by Body Mass Index (BMI), a mathematical calculation, derived from the weight in kilograms divided by the

square of the height in metres (kg/m^2). Body mass index is the most widespread tool for measuring overweight in population and clinical settings. By WHO criteria a BMI of less than 25 is considered normal weight, that between 25 to 29.9 is overweight, 30 or greater is considered obese (WHO, 1997). While these definitions provide common benchmarks for assessment the risks of disease in all populations increase progressively from even lower BMI levels.

According to a report by the National Audit Office (NAO) 65% of English adults were estimated to be overweight and 19% obese (NAO, 2001). The report also shows approximately two-thirds of men and over half of women in Britain are overweight and obese. In the United States 60% of people (about 170 million) are overweight, and 27% of people (about 70 million) are obese and at medical risk (Hedley et al, 2004). However, this prevalence has increased by about 10 –40% in many countries in the past 10 years. The most dramatic increase amongst European countries has been in the UK, where it has more than tripled since 1980.

1.1.2 Consequences of obesity

Obesity has significant co-morbidities and these are associated with substantial health care and social cost. The cost of obesity in Britain was 2.5 billion pounds per year with 30,000 premature deaths in England alone in 1998 (NAO, 2001).

Obesity is a risk factor for type 2 diabetes (Colditz et al, 1995; Chan et al, 1994), cardiovascular disease (Meigs et al, 1997), hypertension (Rocchini, 2004), dyslipidaemia (Despres et al, 2004), sleep apnoea (Strohl et al, 2004),

musculoskeletal disorders (Manson, 2004), degenerative disease (Felson et al, 1988) and some cancers (Manson et al, 1995).

Elevations in body mass increases cardiovascular disease due to elevating blood pressure, cholesterol, triglycerides, and increasing insulin resistance. A 20% reduction in body weight can reduce cardiovascular risk by 40% (Savage et al, 2003). Furthermore this reduced risk can be maintained by keeping BMI within the normal range.

The prevalence of type 2 diabetes is rising dramatically throughout the world and it has been predicted that the number of adults with diabetes will increase by 46% from 151 million in 2000 to 221 million in 2010 (Amos et al, 1997). The incidence of diabetes increases with increasing body weight. Diabetes is three times more likely in obese individuals with a BMI of 28 or greater. Furthermore, developing type 2 diabetes is approximately 40 times more in adults with BMI >35 compared with their peers with a BMI between 18.5 and 24.9 (Colditz et al, 1995).

The risk of certain cancers also increases with increasing BMI (Manson et al, 1995). Other diseases associated with obesity include sleep apnoea, abdominal hernias, varicose veins, gout, gall bladder disease, respiratory problems and liver malfunction (Bray, 2004).

Morbid obesity, indicated by a BMI over 40, is so closely associated with various health problems that it is regarded as a disease in its own right (Laville, 1993). Current trends in our society recognize morbid obesity as becoming an epidemic and leading to a major crisis in the near future. In the United States, 25% of children under the age of 16 are also approaching morbid obesity (Magarey et al, 2003).

The personal economic and social costs of obesity are also significant in terms of reduced quality of life and poor social integration. However, this condition is preventable and treatable in many cases. Therefore, the first aim should be prevention, but it will also be important to develop strategies to treat those already affected with obesity, as even modest weight loss of 5 –10% has been shown to reverse many of the co-morbidities associated with obesity and to result in significant health gains (Bray, 2004).

1.1.3 Causes of obesity

Obesity is a complex, multifactorial, chronic disease that is caused by a combination of both genetic and environmental influences (Speakman, 2004). While obesity has been shown to be due to single gene mutations, such as seen in humans with leptin or leptin receptor gene mutations, these are rare and only constitute a minority of normal obesity (Zhang et al, 1994). Furthermore, despite the identification of over 360 genes that are implicated in obesity, genetic abnormalities on their own cannot explain the rapid increases in obesity reported globally today (Rossner et al, 2002).

Evidence suggests that environmental factors may be more attributable to the current epidemic of obesity (Speakman, 2004). The consequence of increasing food intake and decreasing physical activity results in the accumulation of excess body fat and obesity. Since the 2nd World War western societies have become progressively more affluent. Both men and women are also participating in the work environment and income generation. While this increases personal wealth, it also contributes to lack of free time. Simultaneously, technological advances have resulted in more sedentary lifestyles, at the workplace, at home and in leisure pursuits. The increased

spending power and reduction in free time of the population has given rise to the fast food industry.

Increasing energy intake especially with cafeteria diets, snacking and highly palatable foods that use excess dietary fat often leads to weight gain (Prentice et al, 1995). Higher energy content per gram is provided by fat than protein and carbohydrate. Fat produces fewer satiety signals and is less able to suppress hunger and importantly it has a higher capacity for storage in the body (Blundell, 1993).

Decreasing energy expenditure, especially with modern sedentary lifestyles, appears to be at least as important as high caloric diets in the development of obesity. Modern technologies especially in the past three decades have affected energy usage. Increasing inactivity such as using cars, using lifts, number of hours watching television and working with computers, use of remote controls and mobile telephones and other aspects of technology save on a great deal of energy expenditure over the time (Prentice et al, 1995; Armstrong et al, 1998).

Obesity will develop if individuals fail to match their energy intake to their energy needs. Therefore, according to 'energy balance equation theory' any increase in energy intake (food) or decrease in energy expenditure (basal expenditure, thermogenesis and physical activity), which imbalance the equation, will result in the accumulation of fat in the body (Spiegelman et al, 2001). As an example, 3500 stored calories equals one pound of stored body fat.

The consequence of increasing stored body fat over time is obesity. In other words, obesity is characterised by an excess of adipose tissue.

1.1.4 Body fat distribution in obesity

The perception that obese individuals differ and that it would be useful to distinguish between several types of obesity is not new. In the late 1940s Vague introduced the concept of male (android) and female (gynoid) patterns of fat distribution (Vague, 1947). He suggested that the topography of fat storage and body type were of prime importance in interpreting the health consequences of an obese state and that android obesity carried a greater health risk than gynoid obesity (Vague, 1956). It wasn't until 40 years later that experimental evidence from both human and animal studies showed that the adipose tissue distribution is an important factor involved in the aetiology of type 2 diabetes and cardiovascular disease (Kissebah et al, 1982; Poulliot et al, 1992). The distribution is different between men and women, men being prone to accumulate their excess of energy in the abdominal region, more specifically in the intra-abdominal depot (visceral) whereas women show a selective deposition of adipose tissue in the gluteo-femoral region. This increase in visceral adipose tissue may play a significant role in the aetiology of metabolic complications increasing the risk of type 2 diabetes and cardiovascular diseases (Chan et al, 1994; Onat et al, 2004). Results of the Quebec Cardiovascular Study have shown that the cluster of metabolic disturbances observed among subjects with visceral obesity [hyperinsulinaemia, hyperapolipoprotein B and small, dense low-density lipoprotein (LDL) particles] is associated with a 20-fold increase in the risk of coronary heart disease in a sample of middle-aged men followed over 5 years (Despres, 2001). Selective mobilization of visceral adipose tissue in response to a weight loss program has been noted among viscerally obese patients,

this reduction in visceral adipose tissue being associated with improvements in the lipoprotein-lipid profile and insulin sensitivity (Despres, 2001). The visceral and subcutaneous adipose tissue depots also differ in terms of activity and secretory function (Richelsen, 1991).

1.1.5 Assessment of obesity

To assess obesity properly, body fat content and fat topography need to be determined and compared to those obtained for an individual of a given age and gender class with valid sets of reference values (Shen et al, 2003). This has proven to be difficult in both research and clinical settings. At least three body fat phenotypes are of prime importance; total body fat content, upper body fat and abdominal visceral fat and these need to be evaluated in a laboratory environment, a clinic and population studies.

Body density derived from underwater weighing and converted to a proportion of fat in the body has been considered the gold standard procedure (Heymsfield, 1990). The method assumes that a two-compartment model of body composition to obtain a valid measure of body fat content. This is accurate when the density of fat free tissue is constant but when this component varies such as seen with exercise, ageing and some diseases, estimates of fat content may be slightly unreliable, mainly due to the variation in the conversion of body density to body fat content.

Dual emission X-ray absorptiometry (DEXA) is another method used to estimate body fat content (Kohrt, 1995). While this method can be used to quantify the absolute amount of fat on the trunk, the abdominal region, or any body segment, it cannot distinguish between subcutaneous and visceral fat. Other methods include isotopic dilution to assess body water, body potassium

content to assess skeletal muscle mass, CT scanning and MRI examination at a large number of sites or over the whole body. Abdominal visceral fat can only be measured by CT scan or MRI (Ross et al, 2004). These methods are expensive and require elaborate instrumentation and thus confined for research purposes.

Simpler approaches of assessing obesity include the use of BMI as the surrogate for body fat content, the prediction of body fat from simple anthropometric measurements such as skinfolds and circumferences, and more recently, bioelectric impedance. Waist circumference and skinfolds give reasonable mean values for the prediction of visceral fat but are not very accurate for a given individual (Owen et al, 1999; Clasey et al, 1999). These methods, while less accurate than CT scans or MRI, are more suited for the clinic or the epidemiological research environment.

The number of fat cells can be estimated from measurements of total body fat and the average size of a fat cell. Rapid proliferation of adipocytes occurs soon after birth to 2 years of age, during late childhood and puberty. In some types of obesity a (hypercellular obesity) fat cell number increase 3-5 times above number (normal numbers being around 60 billion) and is associated with early or middle childhood and may also occur in adult life. In people more than 75% above their ideal body weight number of fat cells is usually higher than normal. Adult onset obesity involves enlargement of adipose tissue cells (hypertrophic obesity) and correlates with an android or truncal fat distribution and metabolic disorders such as glucose intolerance, hyperlipidaemia, hypertension and coronary artery disease (Walton, 1995).

1.2 Adipose tissue

1.2.1 Adipose tissue deposition

Adipose tissues differ from many other tissues in that they occur in multiple dispersed sites around the body (Shen et al, 2003). In obesity increased amount of fat is found in the subcutaneous layers between the muscle and dermis as well as fat deposits around the heart, liver, kidneys and other visceral organs. The adipose tissue is considered an important component of the body's system of energy balance and a storage depot. Around fifty percent of the tissue is composed of adipocytes, while the rest comprises of other cell types such as preadipocytes, endothelial cells, macrophages, blood vessels and neurons (Napolitano, 1965; Fain et al, 2002). They are found in the subcutaneous layers, which are located between skin and muscle, and intra-abdominal (visceral), which is distributed around the internal organs (Cinti, 2000). The tissue is composed of two types of cells, brown and white adipocytes, which express and secrete different proteins and vary in function. In humans significant amounts of brown adipose tissue are found only in the neonatal period. However, ordinary white fat may contain small islands of brown fat (Cinti, 1999). The data presented in this report are based upon the study of white adipose tissue.

1.2.2 Adipose tissue in obesity

There is some evidence indicating that the cellular composition of the adipose tissue changes in obesity (Wellen et al, 2003). Evidence exists for significant macrophage infiltration into adipose tissue in obesity (Xu et al, 2003) and may explain the inflammatory changes that occur in the obese state. Clusters of small, nucleated cells are present in obese adipose tissue. These clusters

became larger and more numerous as the animals ages and gains weight. It has been shown that the largest class of genes significantly regulated in obesity, consists of macrophage and inflammatory genes in white adipose tissue (Fig 1.1). Although adipocyte precursors have potent phagocytic capacity and can be transformed into macrophage-like cells, there is the possibility that the cellular source of these inflammatory changes may not be only adipocytes, but also reticuloendothelial cells present in adipose tissue (Weisberg et al, 2003). Experiments in cultured clonal preadipocytes confirmed that the same inflammatory genes identified in the stromal-vascular fraction of white adipose tissue were not expressed in these cells (Xu et al, 2003). Weisberg et al. also provide evidence that macrophage infiltration of adipose tissue is characteristic of human obesity, by determining that both BMI and average adipocyte size were significant predictors of macrophage accumulation in adipose tissue (Weisberg et al, 2003). These data bring the potential involvement of inflammation to the other pathologies associated with obesity. It is likely that a rich array of mechanistic and therapeutic developments will emerge from studies of the inflammatory pathways active in obesity and associated disorders.

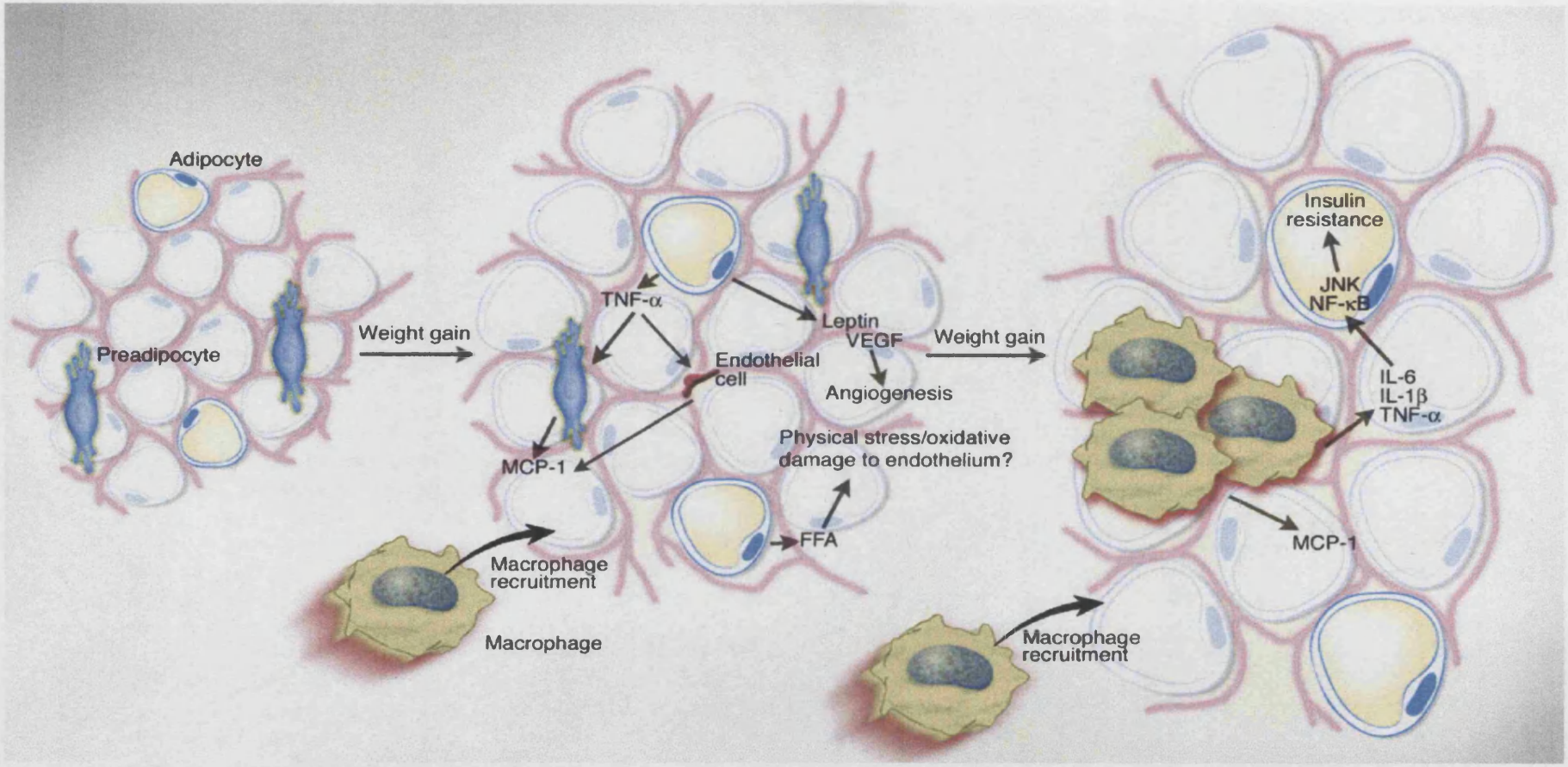


Figure 1.1: Cellular Composition of adipose tissue in obesity

In obesity adipose tissue is characterized by inflammation and macrophage infiltration. In obesity, low level secretion of TNFα by adipocytes can stimulate preadipocytes to produce monocyte chemoattractant protein-1 (MCP-1). Endothelial cells also secrete MCP-1 in response to cytokines. Both preadipocytes and endothelial cells could be responsible for attracting macrophages to adipose tissue. This could perpetuate a vicious cycle of macrophage recruitment, production of inflammatory cytokines, and impairment of adipocyte function (Wellen et al, 2003).

1.3 The study of adipose tissue

The study of adipose tissue is in the main carried out *in vitro* using cell-lines or primary adipose tissue derived cells or in adipose tissue organ cultures. In humans *in vivo* investigation of adipose tissue involves microdialysis or arterio-venous difference studies (de la Pena et al, 2000). All of these techniques have their advantages and disadvantages and these are outlined below.

1.3.1 *In vitro* studies

In vitro studies on human adipose tissue and adipocytes involve organ culture of adipose tissue fragments or primary culture of adipocytes isolated by collagenase digestion of adipose tissue (Rodel, 1964; Fain et al, 2002). Organ culture, as applied to the adipose tissue, refers to intact fragments of adipose tissue placed in a complete, buffered culture medium that contains nutrients and electrolytes. The major strength of this method is the good maintenance of gene expression and adipocyte function within adipose tissue placed in organ culture for upto 2 weeks (Chajek-Shaul et al, 1996). Organ cultures have been used to assess the long-term effects of hormones on the metabolism of human, rat, ovine, bovine, murine and porcine tissue (frohlich et al, 1972; Apple et al, 1992; keys et al, 1992; Baba et al, 1991). In all these species the long-term effects seem to reflect the known *in vivo* effects.

The advantage of primary culture of fat isolated by collagenase digestion over organ cultures is that only adipocytes are present here. Culture of fat cells is different from culture of newly differentiated adipocytes derived from stromal precursors. The latter generally remain multilocular, that is they have multiple lipid droplets, and there is some evidence that these cells are not fully

differentiated. The chief advantage of the study of isolated adipocytes is that they are fully differentiated, unilocular adipocytes (Rodel, 1964).

In addition, there are also murine clonal lines available. Adipocyte precursor cell lines can be segregated into two classes, pluripotent fibroblasts and unipotent preadipocytes (Ntambi et al, 2000). The pluripotent fibroblasts can be converted to preadipose, premuscle and precartilage tissue upon its specific treatment. These multipotent fibroblasts act as good models for understanding the events responsible for cellular determination of the separate cell fates. The unipotent preadipocytes (such as Ob17 and its sub-clone, 3T3-L1 and 3T3-F422A), have undergone determination and can either remain as preadipocytes or undergo conversion to adipose tissue (Ntambi et al, 2000). They are ideal for studying the molecular events responsible for the conversion of preadipocytes into adipocytes. The identification of specific developmental markers have also allowed for the comparison of the developmental programs of the various cell lines. The 3T3-L1 and 3T3-F422A culture lines, derived from disaggregated Swiss 3T3 mouse embryos (Green and Kehinde 1974), are the most widely used culture models. The 3T3-L1cell-line is one of the most well-characterized and reliable models for studying the conversion of preadipocytes into adipocytes. These cells are morphologically similar to fibroblastic preadipose cells found in the stroma of adipose tissue, and once differentiated, they exhibit virtually all of the characteristics associated with adipocytes present within the adipose tissue.

The advantage of using a cell-line is that it produces homogenous population of cells that are all at the same stage of differentiation. This allows for a definitive response to treatments. In addition, these cells can be passaged

indefinitely, which provides a consistent source of preadipocytes for study (Ntambi et al, 2000). Confluent 3T3-L1 preadipocytes can be differentiated synchronously by a defined adipogenic cocktail. Maximal differentiation is achieved upon treatment with the combination of insulin, a glucocorticoid, an agent that elevates intracellular cAMP levels, and cosmic calf serum (Student et al, 1980). Insulin is known to act through the insulin-like growth factor 1 (IGF-1) receptor. Dexamethasone (DEX), a synthetic glucocorticoid agonist, is traditionally used to stimulate the glucocorticoid receptor pathway. Isobutylmethylxanthine (IBMX), a cAMP-phosphodiesterase inhibitor, is traditionally used to stimulate the cAMP-dependent protein kinase pathway.

1.3.2 *In vivo* studies

Two techniques have been utilised for the study of human adipose tissue metabolism *in vivo*: microdialysis, and arterio-venous difference method. Microdialysis enables the concentration, of mainly water-soluble, molecules in the interstitial fluid to be measured (Arner and Bolinder 1991). Arterio-venous studies involve catheterization of the venous drainage from the subcutaneous abdominal adipose tissue (Arner, 1995). Blood obtained by this method shows all the characteristics expected of adipose tissue drainage. The characteristics of this tissue are quite distinct from those of the superficial, mainly skin, or the deep, mainly muscle, tissues of the forearm. The depot studied appears to be typical of adipose tissue as a whole, in terms of non-esterified fatty acid release. In comparison with the microdialysis technique, the arterio-venous difference method allows easier quantification of substrate uptake and release, and enables the study of hydrophobic molecules (e.g. fatty acids, triacylglycerol). On the other hand, it does not allow the study of more than

one depot, or the local introduction of effectors of metabolism (e.g. adrenergic agents) (Frayn et al, 1993).

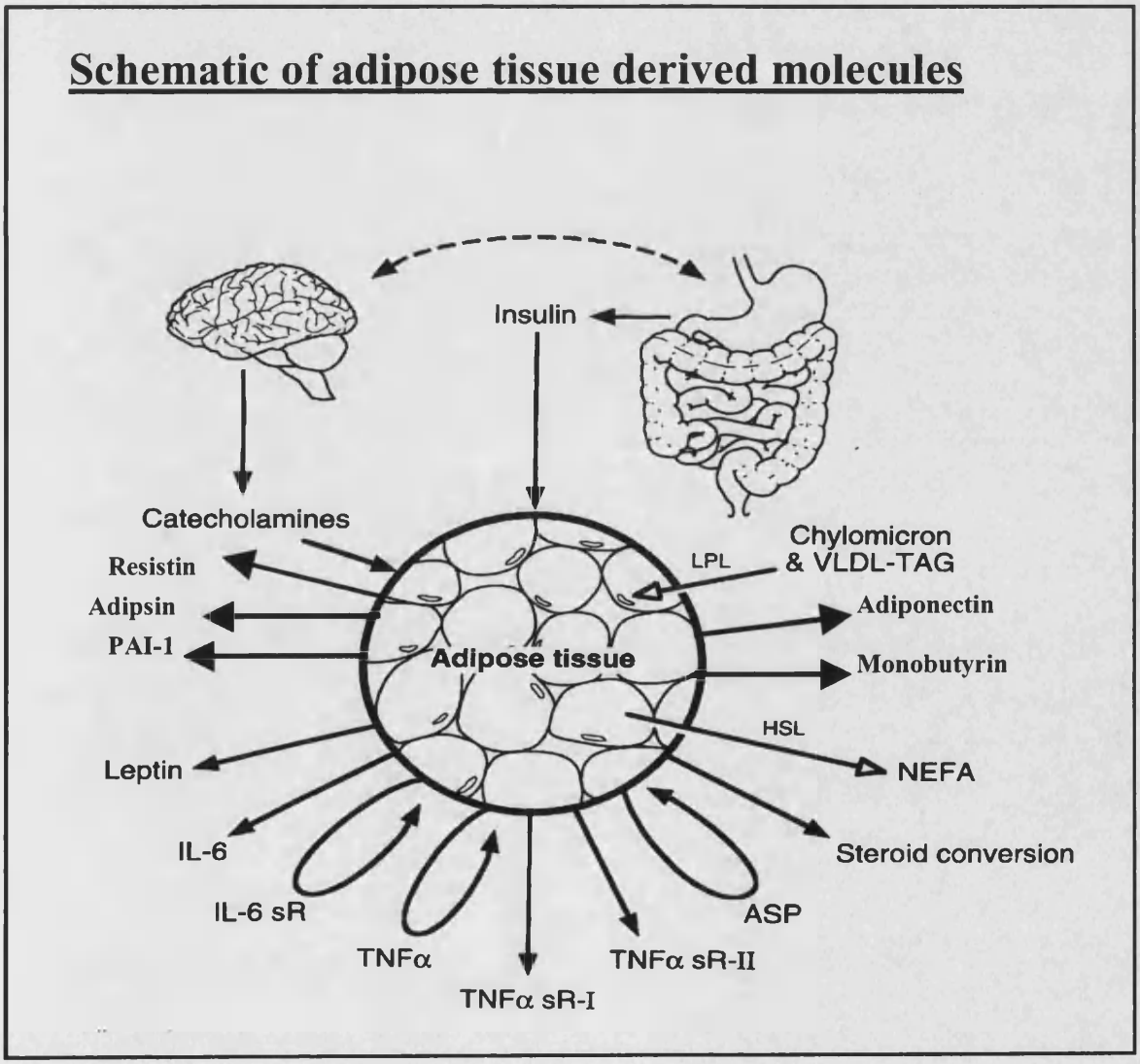
1.4 Adipose tissue derived factors

There has been a long-held belief that accumulation of adipose tissue may lead to increased risk of type 2 diabetes and cardiovascular disease because of associated metabolic alterations. For instance, non-esterified fatty acids (NEFA) are released from adipose tissue and with the increased adipose tissue mass of obesity, plasma NEFA concentrations are almost inevitably raised (Flatt et al, 1972; Opie et al, 1963). Elevated plasma NEFA concentrations may relate either directly or indirectly to other risk factors for cardiovascular disease (Frayn et al, 1996). In addition, as adipocytes enlarge with fat storage, they become less metabolically active and respond less well to insulin. An impaired ability of adipose tissue to respond rapidly to insulin and other hormones could be seen as a factor causing insulin resistance, and ultimately increased cardiovascular risk, through metabolic perturbations (Frayn, 2002).

However, currently, a central integrator of the metabolic programme is considered for adipocyte activity. In addition to the tissue acting as energy storage organ, with triacylglycerols acting as efficient energy reserve, the subcutaneous adipose tissue depots act as thermal insulation. The tissue is also essential for normal glucose homeostasis. The last few years have seen an explosion of research on alternative, non-metabolic links between adipose tissue, insulin resistance and cardiovascular disease. Indeed, current evidence suggests that the association between obesity and cardiovascular disease can be explained, at least in part, by novel signalling molecules,

adipokines, emanating from, or expressed in, adipose tissue. The diversity of these factors includes enzymes such as lipoprotein lipase and hormone sensitive lipase, growth factors and cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) and several other hormone-like molecules involved in metabolism (leptin, adiponectin/Acrp30, resistin and acylation stimulation protein) (Mohamed-Ali et al, 1998)(Figure 1.2).

Figure 1.2: schematic view of biologically active fat-derived mediators



It has been proposed that the secretion of molecules, such as leptin, adiponectin and IL-6 by adipose tissue, combined with the actions of adipose tissue-expressed TNFα in obesity, could underlie the association of insulin resistance with endothelial dysfunction, leading to CHD (Kern et al, 2001). The role(s) and regulation of these adipose tissue-derived molecules have been reviewed extensively and elegantly elsewhere (Mohamed-Ali et al, 1998; Kern et al, 2001) and for the purposes of this project two novel and distinct adipose tissue signals have been investigated with known associations with increased risk of type 2 diabetes and coronary heart disease:

- Interleukin-6 (IL-6) and
- Asymmetric dimethyl arginine (ADMA), the endogenous inhibitor of nitric oxide (NO)

These signals will be discussed below.

1.4.1 Interleukin-6

IL-6 is a proinflammatory cytokine with potent effects in host defence (Miossec, 1991). It is 22-28 kDa and is synthesized as a 212 amino acid (aa) precursor protein, with a 28 aa signal peptide and a 184 aa mature segment (Akira et al, 1993). IL-6 is expressed in adipose tissue and significant amounts of this cytokine are released, largely by the visceral adipose tissue, into the systemic circulation and its plasma levels increase with obesity (Fried et al, 1998; Mohamed-Ali et al, 1997).

1.4.1.1 IL-6 receptors

The biological activities of IL-6 are initiated by binding of the ligand to a single receptor. The IL-6 receptor (IL-6R) comprises two chains, a ligand binding, predominantly extracytoplasmic chain (IL-6R; gp80) and the signal

transducing gp130 chain (Hirano, 1998). The gp130 by itself has little or no IL-6 binding property, but it plays a part in signal transduction (Hirano et al, 1997). The binding of IL-6 to IL-6R is predominantly an extracellular process. This complex, IL-6/IL-6R, can be formed with either soluble or membrane-bound IL-6R (Kishimoto et al, 1992). The binding of IL-6 to IL-6R in the presence of gp130 leads to the formation of high affinity binding sites, gp130 dimerisation and signal transduction (Hirano, 1998). Unlike in the case of TNFα, where the soluble receptors may function as inhibitors for the ligand, both recombinant and naturally produced circulating, soluble IL-6R (sIL-6R) enable cells that express gp130 but not IL-6R to respond to IL-6 (Hirano, 1998). The signal transducing gp130 is abundantly expressed in most cell types, while IL-6R is expressed in a variety of cells in extremely low quantity (Kishimoto et al, 1992).

1.4.1.2 Acute and chronic release of IL-6

In addition to adipocytes/adipose tissue many different cells including macrophages, endothelial cells, smooth and skeletal muscle cells produce IL-6 (van-der-Poll et al, 1994; Purohit et al, 1995; Steensberg et al, 2001; Mohamed-Ali et al, 1998). However, the characteristics and regulation of IL-6 production differs depending on cellular origin. During infection or in response to infectious stimuli, such as lipopolysaccharide (LPS), IL-6 is released from immune cells (*e.g.* monocytes and macrophages). Skeletal muscle cells on the other hand produce physiologically significant quantities of IL-6 in response to exercise (Pedersen et al, 2001).

Both post-exercise and during infection the magnitude of the cytokine response is far greater but of a shorter duration, lasting hours or perhaps days, than that seen in metabolic diseases such as obesity. In obesity there is a chronic, low-level elevation in the circulating IL-6, probably of adipocyte origin (Fig 1.3). The metabolic consequences of acute, as opposed to chronic, elevations in circulating IL-6 levels are quite different, and as yet unresolved.

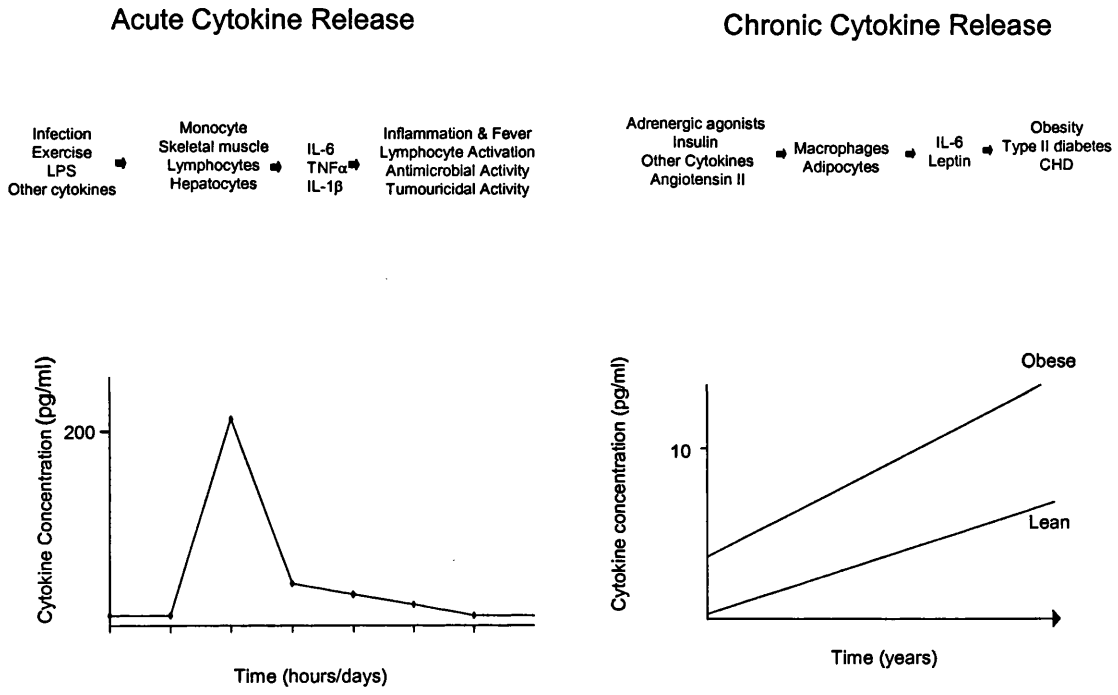


Figure 1.3: Chronic and acute IL-6 release

1.4.1.3 Effects of acute elevation in IL-6

In humans, acute elevation in IL-6 levels, as seen after intravenous administration, is associated with increased plasma glucose clearance rate and hyperinsulinaemia, indicating that IL-6 stimulates glucose uptake *in vivo* (Stouthard et al, 1995). It also increases basal and insulin-stimulated glucose

uptake by 3T3.L1 and 3T3.F442A adipocytes *in vitro* (Stouthard et al, 1996). Stouthard et al. showed infusion of IL-6 to cause elevated serum triglycerides and NEFA concentrations (Stouthard et al, 1996). This hypertriglyceridaemia was caused by stimulation of hepatic triglyceride secretion and was independent of endogenous catecholamines (Metzger et al, 2001). IL-6 has been shown to stimulate insulin release from a hamster islet cell line (Shimizu et al, 2000). IL-6 stimulates glucose and fatty acid oxidation, and induces the release of glucagon and cortisol (Tsigos et al, 1997). IL-6 release from contracting skeletal muscle increases when muscle glycogen availability is reduced and increases glucose uptake. Also during exercise, IL-6 may have immunomodulatory effects, where it inhibits the expression of TNFα while inducing various anti-inflammatory cytokines such as interleukin-1 receptor antagonist (IL-1Ra) and interleukin-10 (IL-10) (Pedersen et al, 2003). IL-6 infusion also increases plasma cortisol that indirectly causes a reduction in lypmphocyte numbers. Thus, collectively the data suggests that acute increases in IL-6 are beneficial, by increasing lipolysis, glucose availability and being anti-inflammatory.

1.4.1.4 Effects of chronic elevation of IL-6

Chronic elevations in circulating IL-6 concentrations are seen with increasing age, in Type 2 diabetes, CHD and obesity; conditions often associated with increases in adipose tissue mass (Goodpaster et al, 2002). In these conditions up to a third of the systemic IL-6 is adipose tissue-derived, with a significant proportion of this production being constitutive (Flower et al, 2003; Mohamed-Ali et al, 1997). That these levels may be adequate to induce the acute phase response may be concluded from elevated CRP also seen in

obesity (McLaughlin et al, 2002). IL-6 has also been shown to inhibit the expression and secretion of the adipose tissue insulin sensitizer, adiponectin (Bruun et al, 2003; Fasshauer et al, 2003). IL-6, produced by adipose tissue, may also affect adipogenesis in a paracrine/autocrine fashion, and play a role in the pathogenesis of obesity. Indirect evidence for a possible role for IL-6 in adipogenesis comes from studies on the senescence accelerated mouse-P6, SAMP 6 (Kajkenova et al, 1997). These animals have been shown to have a higher percent body fat and e*x vivo* bone marrow cultures from these mice exhibited an increase in the number of colony-forming unit adipocytes, as well as an increase in the number of fully differentiated marrow adipocytes. Long-term bone marrow cultures from SAMP 6 produced more IL-6. These data suggest a switch in the differentiation programme and support the existence of a reciprocal relationship between osteoblastogenesis and adipogenesis. This may explain the association of elevated systemic IL-6, decreased bone formation and the resulting osteopaenia with increased adiposity seen with advancing age in both animals and humans.

The data on the effect of IL-6 on lipolysis are also conflicting. In a study looking at the effect of food intake, sympathetic activation and lipolysis, it was reported that while TNFα correlated with lipolysis, IL-6 did not (Orban et al, 1999). Also *in vitro* studies have shown that in 3T3-L1 and 3T3-F442A adipocytes chronic exposure to IL-6 inhibits β-adrenergically stimulated lipolysis (Clarke & Mohamed-Ali, 2003). However, previous reports have shown that IL-6 reduces lipoprotein lipase activity in adipose tissue and increases lipolysis (Mattacks et al, 1999). Therefore, while there is apparently

a physiological difference between acute and chronic elevations in IL-6, the available data are not entirely clear and require further investigation.

1.4.1.5 IL-6 and metabolic disease

IL-6 may play a key role in several mechanisms that contribute to the development of CHD (Fig 1.4) (Yudkin et al, 2000). IL-6 is a powerful inducer of the hepatic acute phase response and several acute phase proteins, such as CRP and fibrinogen, are potent cardiovascular risk factors. Elevated concentrations of CRP are found in patients with acute coronary syndromes, and predict future risk in healthy subjects. Elevated levels of fibrinogen, with autocrine and paracrine activation of monocytes by IL-6 in the vessel wall contribute to the deposition of fibrin.

The acute phase response is associated with increased blood viscosity, platelet number and activity. IL-6 decreases lipoprotein lipase activity and monomeric lipoprotein lipase (inactive form) levels in plasma (Wallberg-Jonsson et al, 1996). Lipoprotein lipase has been shown to mediate the uptake of lipoproteins into cells, with the monomeric form inhibiting the uptake mediated by the dimeric lipoprotein lipase (Krapp et al, 1995). Therefore, the IL-6 mediated reduction in monomeric lipoprotein lipase may increase macrophage uptake of lipids. In fatty streaks and in the atheromatous 'cap' and 'shoulder' regions, macrophage foam cells and smooth muscle cells express IL-6, suggesting a role for this cytokine along with interleukin-1β (IL-1β) and TNFα , in the progression of atherosclerosis.

Circulating IL-6 stimulates the hypothalamo-pituitary-adrenal (HPA) axis, activation of which is associated with central obesity, hypertension and insulin resistance. IL-6 receptors are present in the hypothalamus, which supports a

direct central role for this cytokine. IL-6 stimulates both thermogenesis and satiety, through a range of central effects, including prostaglandin synthesis and corticotrophin releasing hormone (CRH) release (Mastorakos et al, 1993). Thus IL-6 may be involved in the pathogenesis of CHD through a combination of autocrine, paracrine and endocrine mechanisms.

Although obesity increases circulating levels of IL-6, which contribute to some of the maladaptive consequences of obesity, the net effects of chronically increased circulating cytokine concentrations remain to be clarified.

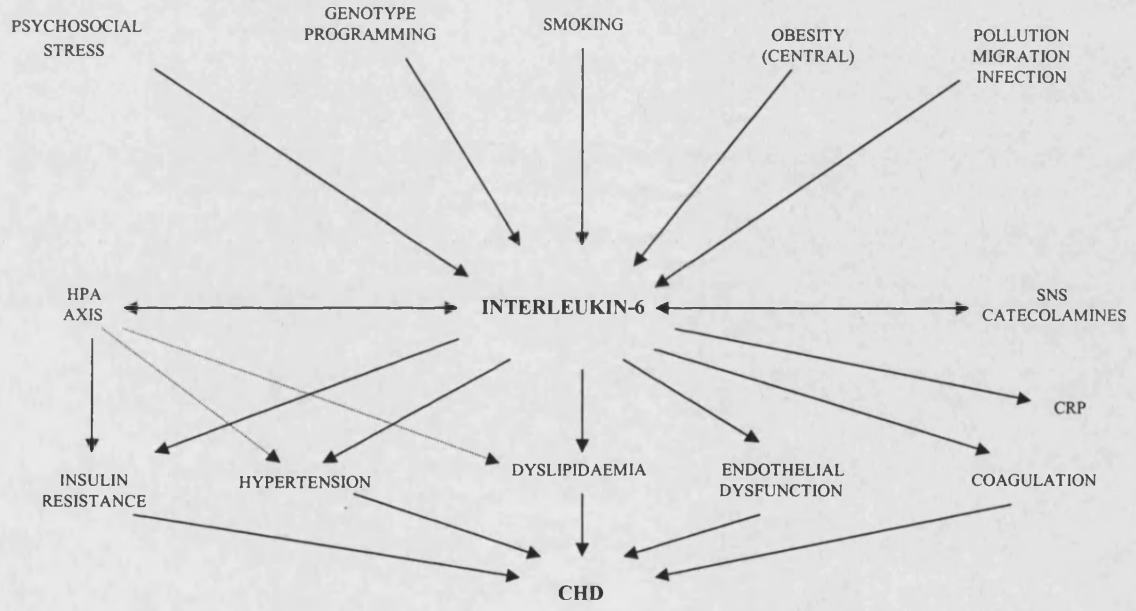


Figure 1.4: Reported correlations of interleukin-6 with CHD risk

Schematic representation of correlations between elevated systemic IL-6 concentrations and various risk factors for coronary heart disease (CHD).It is postulated that IL-6 plays a role in the pathogenesis of CHD through a combination of autocrine, paracrine and endocrine mechanisms. IL-6 is a powerful inducer of the hepatic acute phase response and several acute phase proteins, such as CRP (C-reactive protein). Circulating IL-6 also stimulates the hypothalamic-pituitary-adrenal (HPA) axis, activation of which is associated with central obesity, hypertension and insulin resistance. It also has an effect on sympathetic nervous system (SNS) and catecholamine release (Yudkin et al, 2000)

1.4.1.6 Mechanism of IL-6 secretion in adipocytes

The mechanism that sustains the basal secretion of adipocytes has not been determined. Adipocytes might be sensitive to IL-6-induced IL-6 release. There are data showing increased expression of IL-6 in differentiated 3T3.F442A adipocytes after treatment with IL-6. IL-1β is a powerful stimulus of IL-6 release from several cell types, including adipocytes and preadipocytes. IL-1β is expressed by adipocytes and preadipocytes (Burysek et al, 1996; Auron et al, 1994), but does not appear to be an endocrine signal from adipose tissue *in vivo*. However it is feasible to suggest that IL-1β expression is elevated in obesity and a COX-mediated prostaglandin pathway is responsible for the basal IL-6 release of adipose tissue.

1.4.1.6.a Prostaglandin E2 and COX pathway

Prostaglandin E_2 (PGE_2) modulates the production of inflammatory cytokines including IL-6 (Fieren et al, 1996). There are two cyclooxygenase (COX) enzymes, COX-1 and COX-2 (Gierse et al, 1995). These isozymes are responsible for the production of PGE_2. They are regulated by different mechanisms in the cell, but catalyse identical biosynthetic reactions. COX-1 and COX-2 regulate cytokine synthesis in adipocytes by different roles. IL-6 synthesis is stimulated by production of endogenous PGE_2. This effect is specifically linked to activation of COX-2 and not COX-1. Evidence for prostaglandin involvement in the regulation of IL-6 production comes from studies in various cell types (Takaoka et al, 1999; Fiebich et al, 2001).

In murine and human osteoblast and in human astrocytoma cells PGE2 stimulates IL-6 production. They have shown that IL-1β-induced IL-6 production is initiated by the production of PGE2. IL-1β was found to induce

the production of PGE2 and IL-6 in a dose-related fashion. PGE2 production was induced by lower concentrations of IL-1β than those needed to induce the production of IL-6. Regarding the role of PGE2 in IL-1β-induced IL-6 production, there have been some reports indicating that IL-6 production occurs after the production of PGE2 in other systems (Hinson et al, 1996; Calkins et al, 1988).

In murine peritoneal macrophages increasing release of IL-6 was found to correlate with increases in production of both PGE2 and PGI2, suggesting an autocrine function for these prostaglandins.

In mast cells PGE1 and PGE2 induce IL-6 production and inhibit TNFα production. In human peripheral blood monocytes, NCX4016 (NO-Aspirin), which is COX inhibitor, suppresses production of IL-6. Overall there is a positive association between endogenous PGE2 production and IL-6 release, with animal models also demonstrating that it is a stimulator of IL-6 production *in vivo* (Williams et al, 1997; Ma et al, 1997).

1.4.1.6.b Aspirin and COX inhibition

Aspirin or acetylsalicylic acid was first synthesised by Felix Hoffman in 1897 and has now become the most commonly used non-steroidal anti-inflammatory drug (NSAID) worldwide (Weissmann, 1991; Flower, 1985). Despite its long history and widespread use it was not until 1971 that Sir John Vane identified the enzyme cyclooxygenase (COX) as the target for NSAIDs, including aspirin (Vane, 1971). COX exists in two forms termed COX-1 and COX-2. Both forms release biologically active lipid mediators called prostaglandins but each COX has a distinct function. COX-1 is believed to regulate normal cell function whereas COX-2 is thought to release

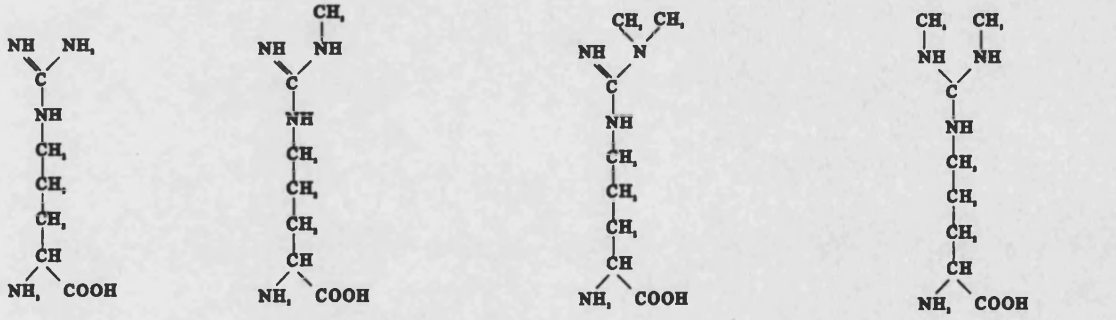
prostaglandins that initiate inflammation, fever and pain. Aspirin has many pharmacological actions (Claria et al, 1995).

The principal mechanism traditionally thought to be responsible for benefit of aspirin is inhibition of prostaglandin synthesis through irreversible acetylation of the COX-1 site (Rothe, 1975). This is the anti-thrombotic action of aspirin and subsequent inhibition of thrombus formation. The anti-inflammatory, anti-tumour and neuroprotective properties of aspirin are less well understood but were believed to involve inhibition of COX-2 activity (Xu et al, 1994). However, aspirin is more active in inhibiting COX-1 activity and inhibits COX-2 activity only at high concentrations. The anti-inflammatory and anti-tumour actions of aspirin are likely to be mediated by mechanisms distinct from direct inhibition of COX activities. Several mechanisms have been reported. One mechanism that has attracted great attention is inhibition of nuclear factor (NF)-κB mediated gene expression (Kopp et al, 1994). However, the concentrations of salicylic acid (SA) and aspirin that are required to inhibit NFκ- B mediated gene expression are in a range that is toxic to humans. It is therefore unlikely that aspirin and SA exert their anti-inflammatory action through NF-κB. Indeed aspirin and SA at such high concentrations have a broad non-specific effect on many cellular proteins. One study has shown that aspirin and sodium salicylate at therapeutic concentrations inhibited the activation of the COX-2 promoter, reduced new mRNA synthesis and suppressed protein expression (Xu et al, 1994). It has been suggested that the therapeutic action of aspirin and salicylates are via suppression of C/EBP mediated gene expression (Saunders et al, 2001). It has also been shown that aspirin, both *in vitro* and *in vivo*, inhibits cytokine synthesis through a variety of molecular mechanisms

(Wu, 2003). These beneficial effects of aspirin were especially significant in subjects with high circulating CRP levels. To date the effect of aspirin at the level of adipose tissue and specifically the adipose tissue release of IL-6 is not known.

1.4.2 Asymmetric Dimethylarginine (ADMA)

Recently, elevated plasma concentration of the naturally occurring NO synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA) has been identified as a risk factor for type 2 diabetes and cardiovascular disease (Boger et al, 1998; Lin et al, 2002; Boger et al, 2004), and the circulating concentration of ADMA correlates closely with the degree of insulin resistance (Stuhlinger et al, 2002). ADMA and the inert isomer symmetric dimthethylarginine (SDMA) are released during the hydrolysis of proteins that contain arginine residues methylated by protein arginine methyltransferases (Clarke, 1993; McBride and Silver 2001) (Fig 1.5).



ARGININE | **L-N MONOMETHYL ARGININE (L-NMMA)** | **ASYMMETRIC DIMETHYLARGININE (ADMA)** | **SYMMETRIC DIMETHYLARGININE (SDMA)**

Figure 1.5: Endogenously produced arginine residues

The primary route of catabolism of ADMA is by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) to form citrulline and dimethlyamine (Ogawa et al, 1989; Kimoto et al, 1995) (Fig 1.6). In contrast, SDMA is excreted unchanged in the urine and is not a NOS inhibitor.

Two isoforms of DDAH have been identified and their expression in several tissues reported (Leiper et al, 1999). The higher the DDAH activity, the lower the ADMA levels and inhibition of DDAH activity results in increased ADMA levels that may reach levels sufficient to inhibit NOS, and alter NO bioavailability.

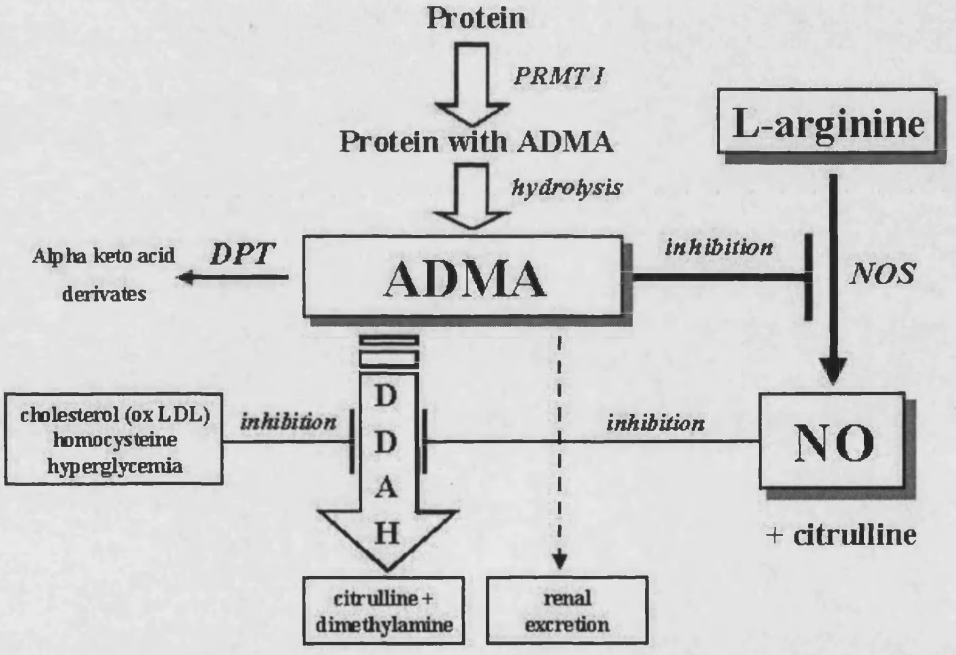


Figure 1.6: ADMA/DDAH pathway

Asymmetric dimethylarginine (ADMA) is an inhibitor of nitric oxide (NO) synthase. ADMA, along with SDMA and L-NMMA, are synthesized by N-methyltransferases, a family of enzymes that methylate L-arginine residues within specific proteins. The primary and specific route of catabolism of ADMA is by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) to form citrulline and dimethylamine. A small proportion of ADMA is also excreted in the urine.

ADMA and SDMA are the major circulating forms of methylarginines in humans and are present in the concentration range of 500nM–1μM in plasma of healthy individuals (Leiper and Vallance 1999).

Given the role of DDAH in the regulation of ADMA degradation, for the second part of this project, we postulated that adipose tissue is a source of ADMA in obesity and interventions that alter adipose DDAH expression would be expected to modulate ADMA levels.

Previous studies have shown that increased plasma levels of ADMA are associated with risk factors for atherosclerosis such as hypercholesterolemia, ageing and hypertension (Voo et al, 2001; Maxwell et al, 1998). It has also been observed that elevated plasma ADMA levels are associated with impaired endothelium-dependent brachial artery vasodilatation in hypercholesterolemic individuals and intima media thickness in healthy subjects and is thought to be an important cause of endothelial cell dysfunction (Boger et al, 1998).

1.4.2.1 ADMA and Cardiovascular disease

ADMA, by blocking NO generation, initiates or encourages atherogenesis, plaque progression and plaque rupture. Some studies have shown that impairment of the NOS pathway independently predicts cardiovascular events (Schachinger et al, 2000; Suwaidi et al, 2000; Gokce et al, 2003).

There are data which show that increased levels of ADMA may contribute to endothelial dysfunction. It has been shown that patients with hypertension (Syrdacki et al, 1999), hyperlipidaemia (Boger et al, 1998), hyperhomocysteinemia (Sydow et al, 2003), coronary artery disease (Valkonen et al, 2001), peripheral arterial occlusive disease (Boger et al,

1997), congestive heart failure (Usui et al, 2001), end-stage renal disease (Kielstein et al, 1999), pulmonary hypertension (Gorenflo et al, 2001) and stroke have elevated levels of ADMA in their plasma.

A causal relationship between increased ADMA levels and endothelial vasodilator dysfunction has been shown in some of these conditions. There is evidence that ADMA regulates vascular resistance in the human as well as the regulation of vascular structure and reactivity.

Infusion of ADMA in healthy volunteers increases blood pressure and vascular resistance and a fall in cardiac output and heart rate occur when its plasma concentration is increased above the physiological range (Achan et al, 2003; Kielstein et al, 2004). Left ventricular hypertrophy is also a feature of prolonged NOS inhibition. ADMA also inhibits angiogenesis in animal models (Jang et al, 2000).

Elevated ADMA concentrations also seem to be a predictor for all-cause and cardiovascular mortality in patients with chronic renal insufficiency and acute coronary events. In non-smoking men with a history of coronary heart disease, those in the upper quartile of ADMA levels had a 4-fold increased risk of an acute coronary event (Valkonen et al, 2001).

1.4.2.2 ADMA and Diabetes

One of the precursors to Type 2 diabetes is insulin resistance, although it is not completely clear why this develops. Elevated ADMA levels have been found in animal models of type 1 and 2 diabetes (Xiong et al, 2003; Paiva et al, 2003). Insulin resistance is associated with raised levels of ADMA. In healthy volunteers without diabetes, ADMA levels were elevated in individuals who were insulin resistant (Stuhlinger et al, 2002). It has been shown that

blood levels of ADMA were directly related to their degree of insulin resistance, independent of other risk factors. However, rosiglitazone a hypoglycemic agent and an insulin sensitizer, with effects largely on adipose tissue, significantly improved sensitivity to insulin and reduced ADMA levels in the blood. Metformin another commonly used oral hypoglycemic agent, with mainly hepatic effects, and some structural similarities with ADMA also reduces systemic levels. The observed reductions in circulating ADMA with both rosiglitazone and metformin therapy could relate to the metabolic changes induced and be a direct consequence of improved insulin sensitivity (Stuhlinger et al, 2002; Vallance and Leiper 2004). Given the metabolic abnormalities of diabetic patients and their high risk for endothelium dysfunction, ADMA could represent an important linking factor for the impaired endothelium-dependent vascular function, insulin resistance and increased cardiovascular risks in Type II diabetes.

1.5 Aims of the study

Two novel cardiovascular risk factors, IL-6 and ADMA, with known associations with increased insulin resistance and endothelial dysfunction were investigated.

The first part of this project investigated the molecular mechanism of adipocyte and adipose tissue IL-6 release.

For this part of the project, the effect of prostaglandins, especially PGE2, and the cyclo-oxygenase (COX) pathway were investigated in the constitutive adipose IL-6 production *in vivo* and *in vitro*.

Additionally, the molecular mechanisms by which COX inhibitors could regulate IL-6 release were examined *ex vivo* in adipose tissue and adipocytes.

The definition of the molecular mechanisms of cytokine secretion would allow more precise targeting of preventive approaches at high-risk individuals who may derive the greatest benefit from preventive treatment and the development of new therapeutic approaches to reduce risk.

Major aims of study on IL-6 release were:

- Effect of COX inhibition on basal secretion
- The prostaglandin receptors and their intracellualr mediators, Ca^{2+} and cAMP, regulating this release.

Other important COX mediated effects were also studied, namely adipogenesis.

The second part of the project set out to investigate the presence of ADMA/DDAH pathway in adipose tissue/adipocyte.

- In cell line
- In adipose tissue and
- Their regulation by diet and pharmacological agents

CHAPTER 2: COX MEDIATED IL-6 SECRETION FROM ADIPOSE TISSUE AND ADIPOCYTES

2.1 INTRODUCTION

There are close associations between interleukin-6 and various risk factors for type II diabetes and coronary heart disease, such as insulin resistance, high triglycerides, low HDL-cholesterol and elevated blood pressure (Yudkin et al, 2000).

IL-6 is expressed in and released by adipose tissue and its levels increase with increasing fat mass (Mohamed-Ali et al, 2000).

Normal circulating IL-6 levels in humans are in a range below 3pg/ml (D'Auria et al, 1997, Yamamura et al, 1998). Infection or injury can drastically, but acutely, increase these levels, up to greater than 200pg/ml (Aikawa et al, 1996). Similar acute elevations are seen after exercise (Febbraio and Pedersen 2002). Plasma levels tend to return to baseline rapidly (<24 hours) once the stimulus is removed. However chronic elevation of IL-6 also occurs, notably in ageing, obesity, diabetes and coronary heart disease (CHD) (Yudkin et al, 2000). Concentrations rarely rise above 10 pg/ml, but levels remain elevated, and may have pathophysiological consequences.

In obesity a significant proportion of the circulating IL-6 is derived from adipose tissue (Mohamed-Ali et al, 2000). At least some of this release appears to be constitutive. The secretion of molecules, such as IL-6, by adipose tissue, could underlie the association between obesity, insulin resistance and endothelial dysfunction, leading to type II diabetes and CHD (Ridker et al, 1997; Ridker et al, 2000; Yudkin et al, 1999). Adipocytes utilise a variety of secretory pathways to release adipokines.

Prostaglandins (PGs), specifically PGE2, modulate production of IL-6 from many cell-types (Williams et al, 1997). The first steps in prostaglandin biosynthesis are catalysed by cyclooxygenase (COX). COX-1 is constitutive and its levels are increased 2 to 4-fold by inflammatory stimuli. COX-2 is inducible and responsible for biosynthesis of inflammatory PGs and its levels increase 10 to 20-fold with inflammation (Katzung, 2001). Expression of PGE2 receptors (EP 1-4) has been shown in adipose tissue. These receptors signal through unique intracellular pathways (Borglum et al, 1999). The EP1 receptor is coupled to phospholipase C and elevates $(Ca^{2+})i$. EP2 and EP4 receptors are coupled to $G\alpha s$ and elevate intracellular cAMP, while the EP3 receptor is coupled to $G\alpha i$ and decreases intracellular cAMP (Hatai, 2003).

2.2 AIM(S)

The aims of this study were to investigate the effect of the cyclo-oxygenase (COX) pathway on basal adipose IL-6 production and the molecular mechanisms involved in this secretion.

2.3 METHODS

2.3.1 Materials

3T3-L1 preadipocytes were a gift from Gokhan S Hotamisligil (University of Harvard, USA). Tri-Reagent was from Sigma (Dorset, UK) and Cambridge BioScience (Cambridge, UK). SYBR-Green reagents for real-time PCR analysis were purchased from Applied Biosystems (Warrington, UK). All oligonucleotides were synthesised by Oswel (Southampton, UK). Real-time PCR were carried out on an ABI-PRISM 7000 (Applied Biosystems). NS-398, PGE2 and the PGE2 receptor agonists (sulprostone, butaprost and PGE1

alcohol) were obtained from Cayman and aspirin (acetyl salicylic acid) from Sigma chemical.

2.3.2 Adipocyte culture

3T3.L1 preadipocyte cell-lines were used in the preliminary experiments. The 3T3.L1 cells were derived from the Swiss 3T3 cell-line prepared from desegregated 17-19 day-old Swiss 3T3 mouse embryos. 3T3.L1 preadipocytes were seeded onto 6-well plates (Corning, High Wycombe, UK) at $6x10^4$ cells/well. Cells were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, UK) supplemented with 10% bovine calf serum (BCS; Perbio, Paisley, UK), 200 units/ml penicillin and 50µg/ml streptomycin, at 37°C/10%CO_2. At confluence (day 0) cells were differentiated by maintaining in DMEM/ cosmic calf serum (CCS, HyClone, Utah, USA) supplemented with insulin (5µg/ml), dexamethasone (1µM) and isobutylmethylxanthine (0.5mM) for 72h. At this stage the cells appeared rounded and contained tiny lipid droplets. The medium was then changed and cells allowed differentiating in DMEM/CCS containing only insulin (5µg/ml) until fully differentiated. The medium was changed every other day until >80% of the cells were differentiated.

2.3.2.1 Intervention

Differentiated adipocytes were exposed to different doses of non selective COX inhibitor, aspirin (5, 2, 1, 0.2 mM), or COX-2 selective inhibitor, NS-398 (0.01, 0.1 and 1). These interventions were done in the presence or absence of IL-1β (10ng.ml^{-1}; IL-1β R&D Systems, Oxon, UK). After 24 h of treatment, culture media were collected to measure IL-6 or PGE2 levels. Cells were also collected for extraction of protein and RNA.

Adipocytes were exposed to a variety of PGE2 receptor agonists: Sulprostone as an EP1/3 agonist, Butaprost as an EP2 agonist and PGE1 alcohol as an EP2/3/4 agonist at 1μM. Following 24 h exposure, media of cells were collected for analyses, as before.

2.3.3 Organ culture of adipose tissue

Subcutaneous and epididymal adipose tissue was removed from 12-week-old C57BL6/J mice animals. Subcutaneous adipose tissue was removed from dorsolumbar area, beneath the skin and without entering the abdominal cavity. Epididymal adipose tissue was removed from epididymal area in the intraabdominal cavity. The tissues were washed in PBS, cut into small pieces and weighed before the experiment.

2.3.3.1 Intervention

0.2g of tissue was incubated in 1.5 ml serum-free media (Cellgro, Hyclone, USA) for 5,10,24 and 48 hours at 37°C / 5% CO_2 in the absence of any additives (as a basal control) or with added COX inhibitors (aspirin: 0.2, 1, 2 and 5mM; NS-398: 0.01, 0.1 and 1μM). At the end of the incubation, media was removed and retained for assay and the tissue was snap frozen in liquid N_2 and stored at –80°C for protein extraction.

2.3.4 Assays

Supernatants from cell culture and explants were assayed using a commercially available IL-6 ELISA (R&D Systems, Oxon, UK) for interlukin-6 and EIA (Cayman) to measure PGE2.

The results from the assay were divided by the weight of fat tissue to give the amount of IL-6 release per gram of tissue.

2.3.5 Cell lysate preparation, protein estimation and SDS-PAGE

2.3.5.1 Cell lysate preparation

Protein was isolated from 0.2g of frozen adipose tissue. The tissue was crushed, digested and homogenised with RIPA buffer (1x PBS, containing 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 supplemented with protease inhibitors cocktail tablet (1tablet/10ml) (Complete, Roche Diagnostic Gmbh, Mannhein, Germany). Protease inhibitor contained 3.0mg Antipain-HCl, 0.5mg Bestatin, 1.0mg Chymostatin, 3.0mg E-64, 0.5mg Leupeptin, 0.5mg Pepstatin, 3.0mg Phosphoaramidon, 20.0mg Pefablock SC, 10.0mg EDTA and 0.5mg Aptotinin. After 15 minutes centrifuging at 14000rpm, the infranatant was recovered and stored at –80°C for protein estimation and SDS- PAGE analysis.

2.3.5.2 Protein estimation

Lowry method was used to estimate total protein content (Tornqvist et al, 1975). Standard samples were prepared in buffer with BSA at concentrations of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml and 0.0313 mg/ml. 5 μl of sample or standard were added to a 96-well plate. After 15 minutes incubation with Bio-Rad protein assay reagent A, 200 μl reagent B were added. They were incubated for a further 15 minutes. Absorbance was measured at 650 nm using plate-reader (Spectra Max 250).

2.3.5.3 SDS-PAGE analysis.

Sodium dodecil sulphate (SDS) polyacrylamide separating gel was used. Separating gel was prepared with 10% polyacrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.05% ammonium persulphate and 0.05% TEMED. The gel

was added by a stacking gel containing of 5% polyacrylamide, 0.125 M Tris-HCl pH 6.8, 0.1% SDS, 0.05% ammonium persulphate and 0.05% TEMED.

25 μg of sample was added in loading buffer (6% SDS, 6% 2-mercaptoethanol, 40% sucrose, 0.2% bromophenol blue in 0.125 M Tris-HCl, pH 6.8). The mixture was boiled for three minutes before loading onto the gel.

Running buffer (20 mM Tris-base, 200 mM glycine and 0.1% SDS) was added into the electrophoresis tank. Then the gels were run at 80 Volt for approximately 90 minutes until enough separation had occurred.

2.3.6 Western analysis

After removing the gel, it soaked in transfer buffer (20 mM Tris-base, 200 mM glycine plus 200ml methanol of total of 1000ml distilled water) for 20 minutes. PVDF membrane was activated by incubation in methanol, distilled water and transfer buffer for 5 seconds, 5 and 10 minutes respectively. Protein was transferred from the gel to the membrane by using semi-dry blot method (Multiphor II, Pharmacia).

16 sheets of filter paper were soaked in transfer buffer prior to transfer. Eight of them were placed on the negative electrode, followed by PVDF with the gel on the top of them. This assembly was sandwiched with a further eight sheets of soaked filter paper covered on the gel, before the positive electrode was placed upon them.

The gels were blotted electrophoretically onto PVDF membranes at the voltage of 25 Volt for 25 minutes. Following the blotting process, the membrane was blocked with 5% non-fat dried milk in PBST (Phosphate Buffer Salin plus 0.1% Tween 20) for one hour at room temperature and washed

three times in PBST at 10 minutes per wash. The blots were probed overnight at 4°C with commercially available rabbit polyclonal antibodies against COX-2 (Cayman) diluted 1:5000 in PBST milk.

The membrane was washed three times in PBST (10 minutes per wash) and secondary antibody consisting of goat anti-rabbit IgG conjugated to horse-radish peroxidase (Amersham Bioscience) was added at 1:5000 dilution in PBST and incubated for one hour at room temperature. The membrane was washed four times and incubated in enhanced chemiluminescence (ECL) plus reagent for two minutes. Antigen-antibody complexes were detected by chemiluminescence with an ECL kit and blots exposed from five seconds to two minutes to high performance chemiluminescence film (Kodak).

2.3.7 RNA isolation

RNA was isolated from cultured adipocytes. Tri-reagent (400 µl to each well of a 6-well plate or 1 ml to 25 cm^2 flasks) was added, mixed and incubated at room temperature for five minutes. Repeated pipetting was used to homogenise the cells. 200 µl DEPC-chloroform per 1 ml Tri-reagent was added. Samples were mixed by vortexing and incubated on ice for five minutes. Samples were then centrifuged at 12000 x g for 15 minutes at 4°C and the aqueous layer removed.

A $^1/_{10}$ volume of DEPC-isopropanol was added, vortexed and centrifuged at 12000 x g for 10 minutes at 4°C. The upper layer was removed to another tube and DEPC-isopropanol was added (500 µl isopropanol per 1 ml Tri-reagent). Samples were incubated overnight at –20°C, and subsequently centrifuged at 14000 x g for 30 minutes at 4°C. At this stage, a small pellet was visible in each tube. All liquid was removed and the pellet washed in

800µl of 75% DEPC-ethanol. Following further centrifugation at 14000 x g for 10 minutes at 4°C the pellet was air-dried and reconstituted in 50 µl nuclease-free water.

2.3.8 cDNA synthesis

4 µl of the sample was diluted in 1 ml DEPC water to measure OD_{260} and OD_{280}. DEPC-water was used as a blank for each measurement. At OD_{260} an optical density of one corresponds to approximately 40 µg/ml RNA in an undiluted sample. Therefore, with the above dilution, an OD of one would indicate 10 mg/ml. Thus, multiplication of the measured OD_{260} by 10 provides the RNA concentration in µg/µl. Purity of the RNA preparation was assessed as a ratio of OD_{260}/OD_{280}, where a value of two indicated pure RNA, and a reading between 1.7 and 2.1 was considered acceptable purity.

5 µg RNA was used to synthesise complementary DNA (cDNA). To 5 µg RNA, a mixture of 7 µl 5x reverse transcriptase buffer, 3.5 µl 0.1M DTT, 2 µl 20 mM dNTP, 1 µl 40 U/µl RNAse inhibitor and 0.5 µl 50 pmol random oligonucleotide primers were added. Total volume of 33 µl was prepared by using nuclease-free water. The mixture was heated to 65°C for 10 minutes. Then, 2 µl reverse transcriptase (200 U/µl) was added on ice, and the sample incubated at 42°C for 90 minutes. cDNA samples were stored at –20°C until required for PCR analysis.

2.3.9 Taq-man Real-time PCR Analysis

Each primer sequence for Taq-man® PCR analysis was designed by using the software package Primer Express. Primers with the concentration of 80 nmol/ml were prepared, and forward and reverse primers were mixed equally

to give a final concentration of 40 nmol/ml of each primer. Reconstituted primers were stored at –20°C.

25 μl total volume of samples per well was run in duplicate. This volume consisted of 1.25 μl specific primer, 12.5 μl SYBR-green solution, 1 μl cDNA and 10.25 μl nuclease-free water. A negative control consisting of nuclease-free water in place of cDNA for each primer was included in PCR reaction. GAPDH as a control mRNA was also run in duplicate. A Standard procedure specified by the ABI Prism 7000 software was used for reactions. Amplification conditions using this method remain constant independent of the primer sequences used.

Data were obtained from a minimum of at least five separate experiments. Data analysis was done by using the $2^{-\Delta\Delta Ct}$ method which was described by Livak and Schmittgen for relative quantification of real-time PCR (Livak and Schmittgen 2001). The data are expressed as changes in expression compared to an arbitrary baseline (described for each individual message investigated).

2.3.10 Plasma membrane Ca channels

This part of experiment was done in collaboration with Dr Gui-ping Sui in Institute of Urology at University College London.

2.3.10.1 Electrophysiological recordings

Membrane currents were recorded by voltage-clamp in the whole-cell configuration, using an Axopatch 1D system (Axon Instruments Inc.). Fire-polished glass pipettes (3-5 MΩ) were used back-filled with a Cs^+-based solution to suppress outward K^+ currents (see below). Cells were superfused with oxygenated Tyrode's at 36°C at a flow rate 1.0ml/min. Voltage-clamp

pulses were generated and data were captured using a Digidata 1200 (Axon Instruments) interfaced to a computer. Currents were sampled at 2 kHz and low-pass filtered at 1 kHz. No leak subtraction was used. Cell capacitance was recorded on membrane rupture with the patch pipette. Data were analysed using the pClamp 8.0 system (Axon Instruments). Cells were held at either –100 or –40 mV for 2 seconds, before 800 ms step pulses were applied from a pre-conditioning pulse at –60 mV for 1 ms. Step pulses were between –100 and +50 mV in 10 mV increments

Peak inward current magnitude was plotted as a function of test potential to generate current-voltage (*i–v*) relationships. The reversal potential, V_r, was calculated by extrapolating the right-hand limb of the linear portion of the *i–v* relationship to the voltage axis (see figure 2.4). The slope of the line was used as an estimate of maximum conductance, g_{max} (units *S*, Siemens). All current and conductance values are normalised to unit cell capacitance. Channel conductance, *g*, at more negative voltages was calculated from $g = i/(V-V_r)$. Activation curves were fitted to the Boltzmann relationship $g/g_{max}=1/(1+\exp(-(V-V_{0.5})/k_d))$; where $V_{0.5}$ is the test potential when $g = 0.5g_{max}$, V_d and k_d are constants. The inactivation kinetics of peak inward current were investigated by holding the cell at voltages between –120 to +50mV, at 10mV increments, for 2 seconds, and applying a test pulse from –60 (1ms) to the voltage at which maximum inward current was attained for each cell (between –30 or –10 mV) for 800ms. The relationship between i/i_{max} and holding potentials of V_h was fitted to the relationship: $i/i_{max} =1/(1+\exp((V_h-V_{0.5})/k_f))$. i_{max} was attained at the most negative holding potentials, $V_{0.5}$ is the

membrane potential when $i = 0.5i_{max}$, k_f is a constant. All data values are given as mean ±SD.

Cells were superfused with Tyrode's solution (mM): NaCl 118; $NaHCO_3$ 24; KCl 4.0; $MgCl_2.6H_2O$ 1.0; $NaH_2PO_4.2H_2O$ 0.4; glucose 6.1; Na pyruvate 5.0; pH 7.4±0.03 gassed with 5% CO_2 & 95% O_2. For Ca-free Tyrode's solution, $CaCl_2$ was replaced by equimolar $MgCl_2$ plus 0.12 mM EGTA. $NiCl_2$ was stored as a 100 mM stock solution in de-ionised water and diluted in Tyrode's solution. Patch pipettes were filled with (mM) CsCl 20; aspartic acid 110; $MgCl_2.6H_2O$ 5.45; Na_2ATP 5.0; $Na_4GTP.2H_2O$ 0.3; EGTA 5.0, HEPES 5.0; pH 7.1 adjusted with CsOH. All chemicals were obtained from Sigma.

2.3.10.2 Fluorescence Confocal imaging

Intracellular Ca^{2+} release, at the single cell level, was measured using standard $[Ca^{2+}]_c$ fluorimetry and imaging techniques to explore the $[Ca^{2+}]_i$ signalling pathways in differentiated adipocytes. Fluorescence imaging, on the confocal system (Zeiss 510 LSM), with calcium indicators (fluo-4) was used to image $[Ca^{2+}]_i$ signals generated. Differentiated 3T3.L1 adipocytes were loaded with Fluo-4 (0.25nM) with 2% pluronic (detergent). Cells were washed and ATP (1-100 μM) or IL-1β(10ng/ml) added.

2.3.11 Statistical Analyses

Data were analysed using the SPSS version 6.0 statistical package (Statistical Package for the Social Sciences, SPSS UK Ltd, Chertsey, UK). Data are presented as means and standard error of means or interquartile ranges. Significance was defined as 2-tailed $p<0.05$.

2.4 RESULTS

2.4.1 COX expression and IL-6 release in adipose tissue organ cultures

In organ cultures of mouse adipose tissue from sub-cutaneous (dorsal-lumbar) and visceral (epididymal) depots COX-2 protein was constitutively expressed and remained elevated for up to 48h, with levels highest at 24h of incubation (Figure 2.1a). Culture media surrounding these tissues showed significant accumulation of IL-6 with a similar time course to the expression of COX-2, with the highest levels after 24 hours (Figure 2.1b).

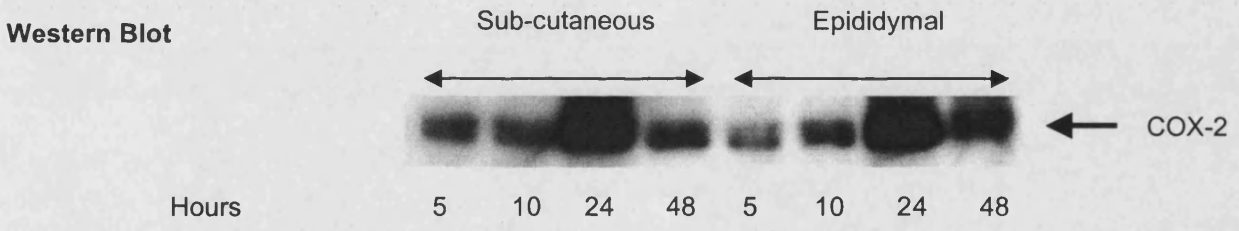


Figure 2.1a: Time course expression of COX-2 protein in subcutaneous and epididymal adipose tissue

Constitutive COX-2 protein expression in sub-cutaneous and epididymal murine adipose tissue explants was seen by western blot analysis. Expression remained elevated for up to 48 hours, with it's a peak at 24 hours.

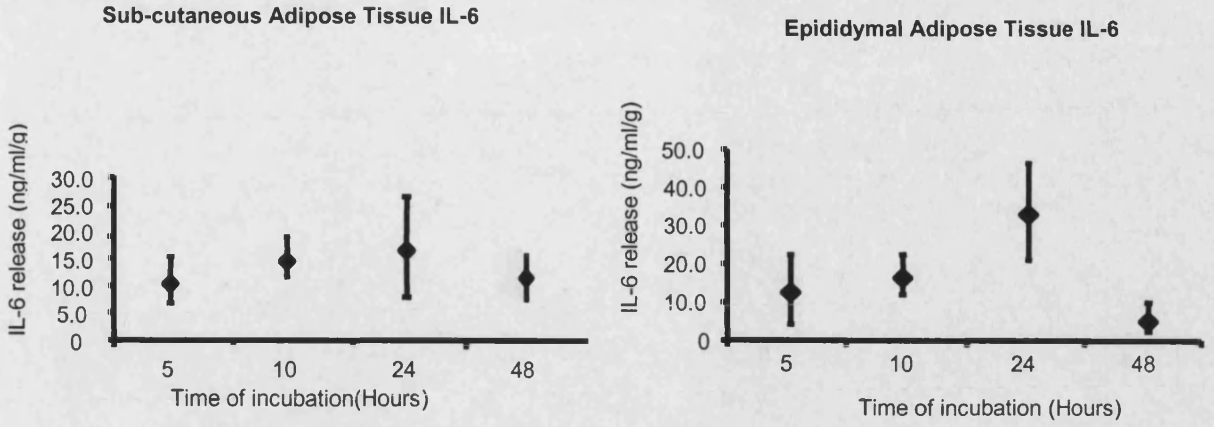


Figure 2.1b. Time course release of IL-6 in subcutaneous and epididymal adipose tissue

IL-6 release from sub-cutaneous and epididymal murine adipose tissue explants up to 48 hours with its peak at 24 hours. The release of IL-6 was calculated based on per gram release of tissue. Data shown as mean±SD, n=12

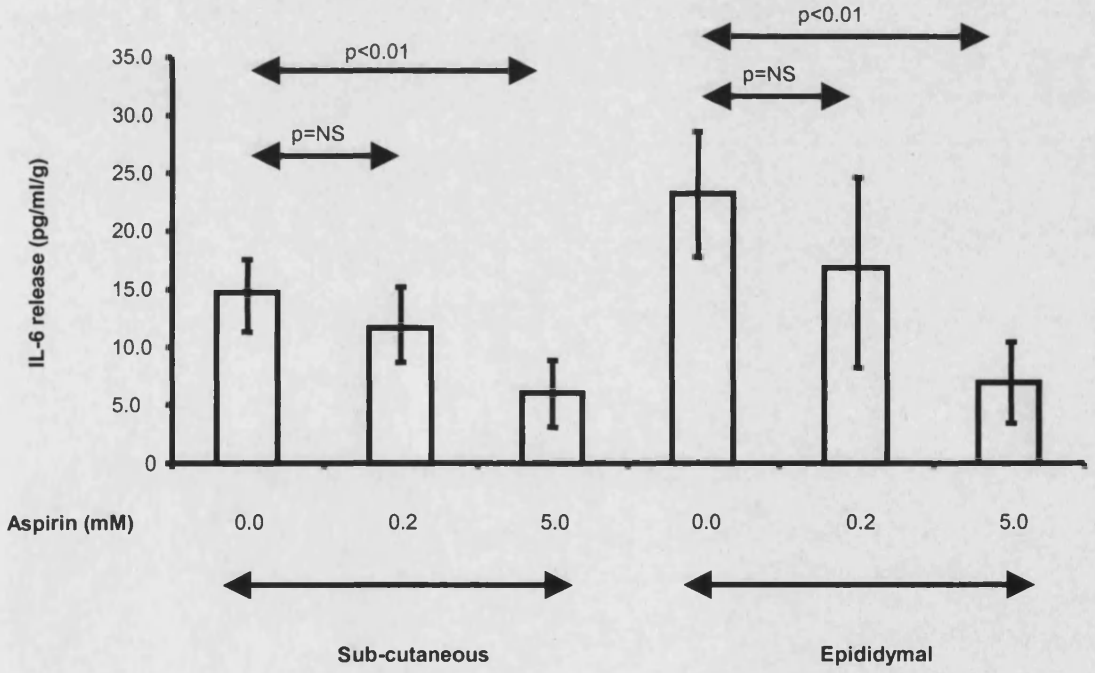


Figure 2.2: Effect of aspirin on IL-6 release in subcutaneous and epididymal adipose tissue explants

Figure 2.2 shows that after 5 hours incubation the IL-6 release from both sub-cutaneous and epididymal adipose tissue was decreased significantly by the non-selective COX inhibitor, aspirin at 5 mM, but not at 0.2 mM.

Furthermore, a selective COX-2 inhibitor, NS-398 (1 μM), also reduced release (by 34.0 – 62.0 %, n=8, p <0.05) (Fig 2.3)

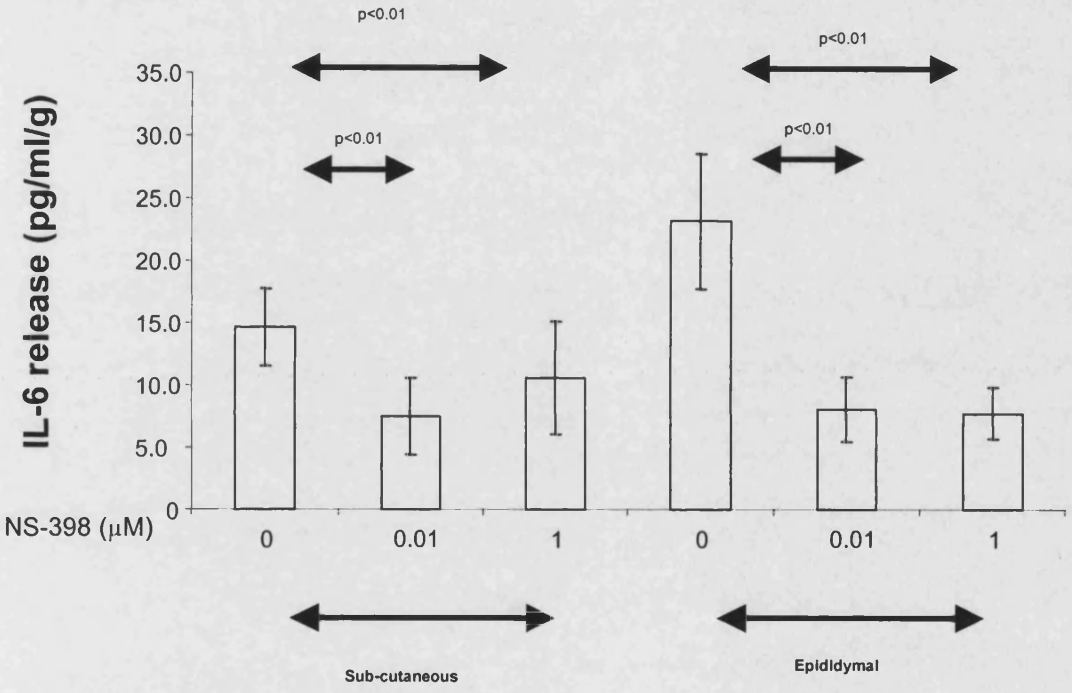


Figure 2.3: Effect of NS-398 (COX-2 selective inhibitor) on IL-6 release in subcutaneous and epididymal adipose tissue explants

Figure shows dose dependent inhibitory effect of a selective COX 2 inhibitor (NS 398, 0.01and 0.1 μM) on IL-6 release in murine subcutaneous and epididymal adipose tissue explants. (Data are presented as mean±SD, n=12)

2.4.2 IL-6 release from differentiated 3T3-L1 adipocytes

In differentiated adipocytes IL1β increased IL-6 release (Flower et al, 2003; Crofford, 1995) and induced the expression of COX-2 protein. In these cells aspirin inhibited the induction of IL-6 release (0.2, 1.0 and 2.0 mM) (Figure 2.4), with higher doses of aspirin (2.0mM) being associated with a greater reduction in IL-6 release compared to low doses of aspirin (0.2 and 1.0mM).

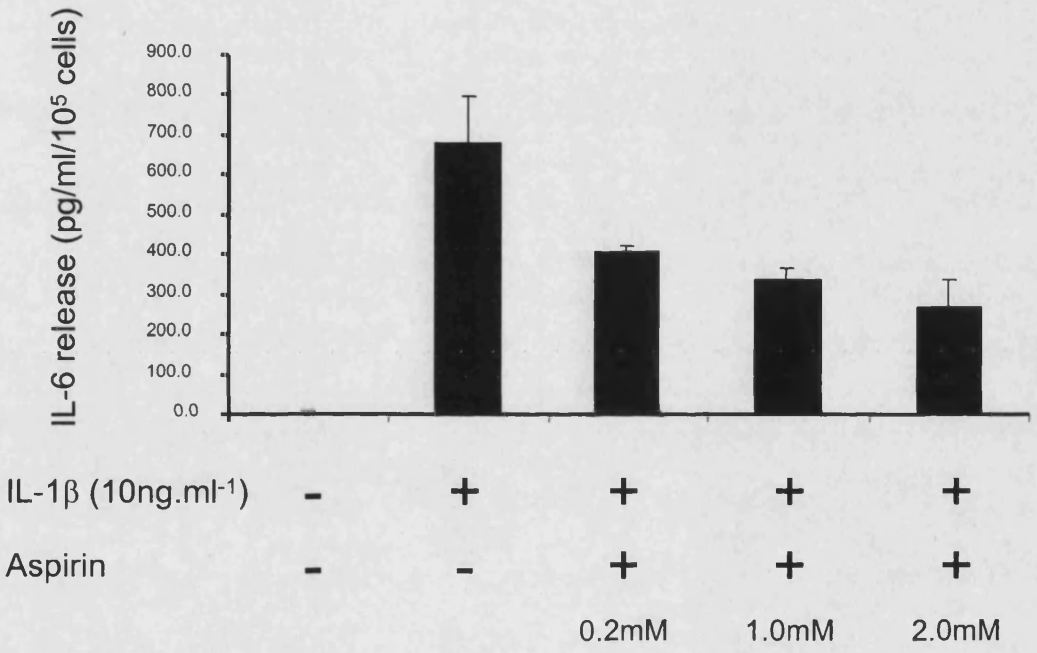


Figure 2.4: Effect of aspirin on IL-6 release of IL-1β induced 3T3-L1 differentiated adipocytes

Dose dependent inhibitory effect of aspirin (0.2, 1.0 and 2.0 mM) on IL-1β-induced (10 ng/ml) IL-6 release in differentiated 3T3L1 adipocytes (data shown as mean±SD; n=12).

2.4.3 PGE2 induced IL-6 release in 3T3-L1 adipocytes

In both 3T3L1 and in adipose tissue explants PGE2 accumulated in the media over a 24h time period, with a positive, significant correlation between IL-6

and PGE2 concentrations ($r=0.67$, $p<0.01$, $n=11$ sample pairs), as seen previously by others (Fain et al, 2001; Fain et al, 2002).

Exogenously added PGE2 also elicited a significant IL-6 output that was both dose and time-dependent (figure 2.4). Peak release of IL-6 from adipocytes was observed following 24 hours incubation. There was significantly higher release of IL-6 in cells treated with 1 and 2 μM PGE2 compared to those with lower concentrations (0.01 and 0.1μM). This effect was evident at all time points.

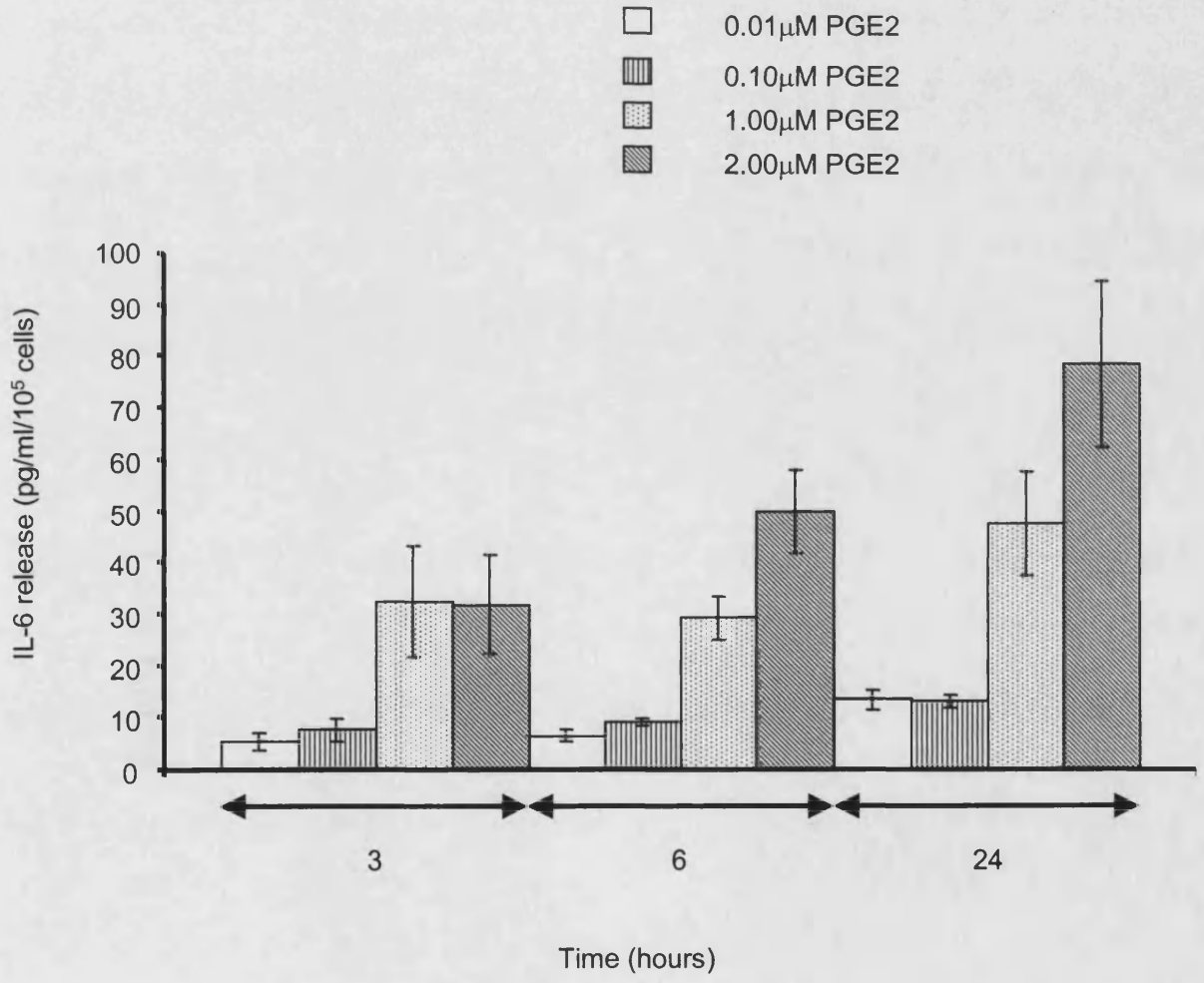


Figure 2.5: Effect of PGE2 on IL-6 release of differentiated adipocytes in a dose and time-dependent manner

2.4.4 Intracellular mediators of PGE2 signalling - Ca^{2+} and cAMP-dependent pathways

PGE2 signalling could be mediated by several EP receptors that either elevate $[Ca^{2+}]_i$ (EP1), or modulate intracellular cAMP levels (EP2, 3 and 4). First, the routes of Ca^{2+} regulation in differentiated 3T3-L1 adipocytes were characterised to determine if Ca^{2+}-sensitive pathways may regulate IL-6 release.

Ca^{2+} currents were measured under voltage-clamp in 18 cells. Large transient inward currents were seen when a cell was depolarised from –100 to between –50 and 0 mV, using a Cs^+-filled pipette (Figure 2.6A). The current was reversibly abolished when extracellular Ca was reduced. The inward current-voltage (*i-v*) relationship is shown as the test potential was varied from a holding potential of –100 mV in 10 mV steps to +50 mV (Figure 2.6B). Current peaked at –40 to –30 mV and reversed at about +50 mV. Shown also is the *i-v* from a holding potential of –40 mV when no inward current was recorded on further depolarisation.

The voltage-dependence of inward current is similar to that of T-type Ca^{2+} channels recorded from other cell types and the lack of inward current at a holding potential of –40 mV (figure 2.6B) suggest an absence of L-type Ca^{2+} current. T-type channels are blocked by sub-millimolar concentrations of Ni^{2+} and this was tested in these cells. Figure 2.6C shows that the inward current was reversibly abolished by 300 μM $NiCl_2$. Finally the voltage-dependence of current activation and inactivation were determined (see Methods) to characterise further the currents. Figure 2.6D shows averaged curves with half-maximal voltages of activation and inactivation of -48.0±0.2 mV (n=14) and -72.9±0.3 mV (n=15) respectively.

The expression of T-type Ca channel was confirmed in 3T3L1 preadipocytes and differentiated adipocytes at the mRNA level, by using Taq-man real time PCR. Both preadipocytes and differentiated adipocytes expressed T-type Ca channels.

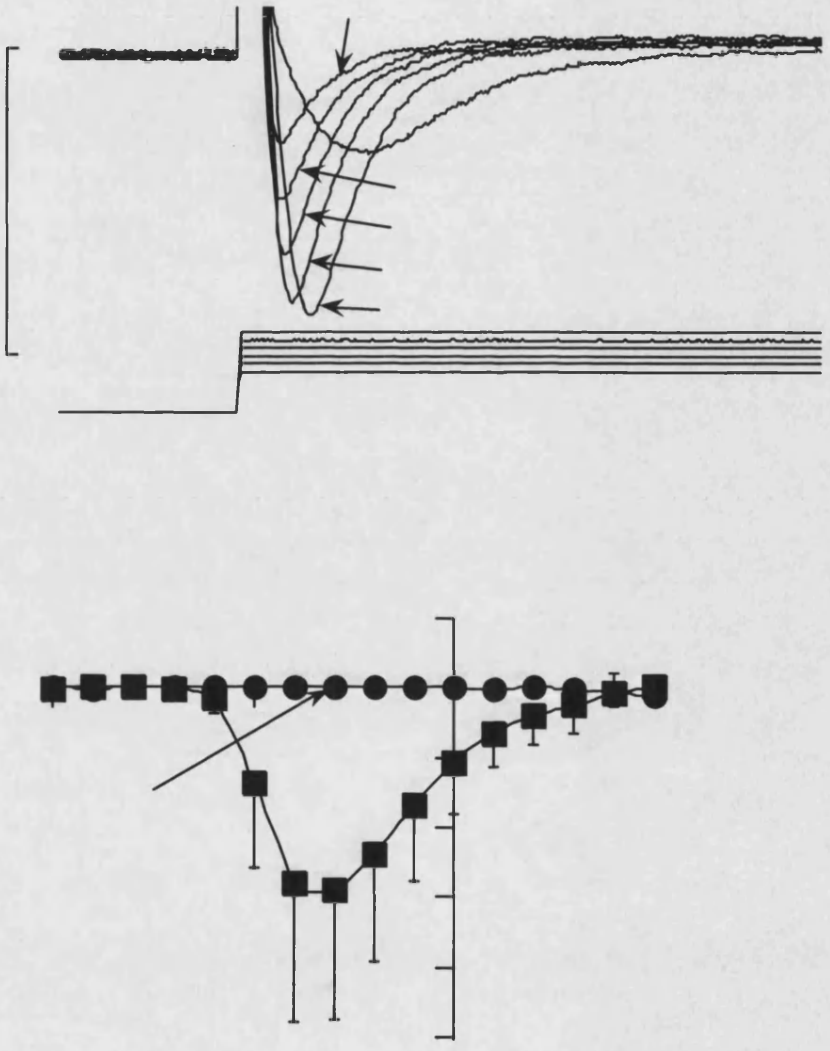


Figure 2.6: Inward currents recorded in isolated 3T3-L1 cells

Figure 2.6A: Inward currents recorded from a holding potential of –100 mV to values between –50 and 0 mV, using a Cs^+-filled patch pipette.

Figure 2.6B: current-voltage relationships of the inward current recorded from a holding potential of either –100 mV (squares) or –40 mV (circles).

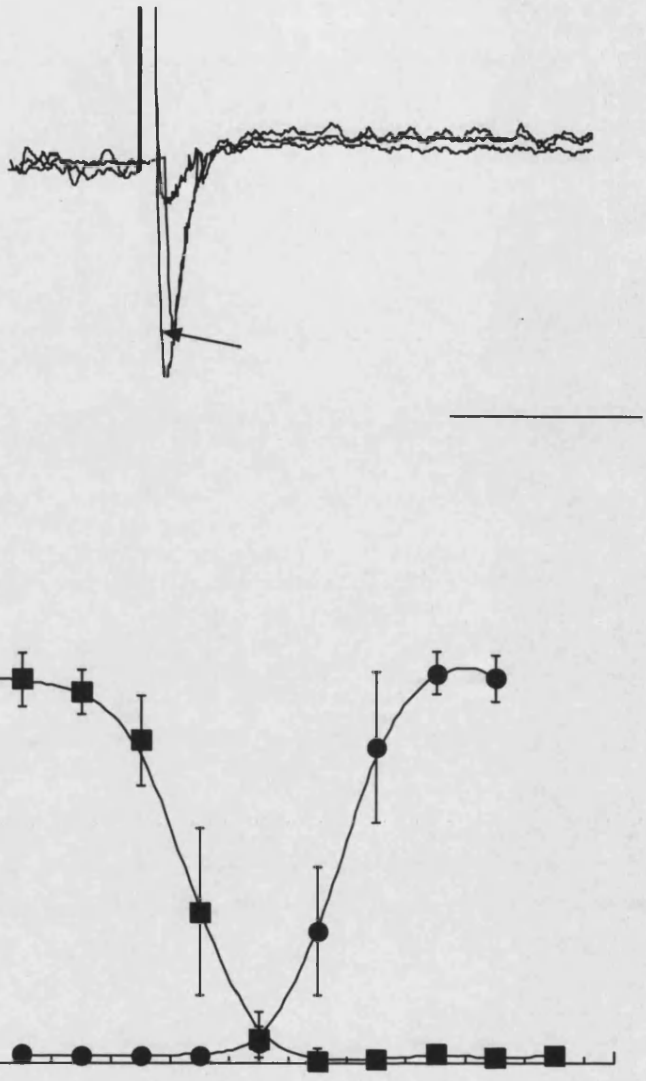


Figure 2.6C: Inward current recorded from a 3T3-L1 cell after five minutes exposure to 300 μM $NiCl_2$: control traces before exposure and 10 minutes after washout from $NiCl_2$ are also shown. Step depolarisation from –100 to –30 mV.

Figure 2.6D: Activation (circles) and inactivation (squares) curves for the inward current. Curves were fitted to the equation in the Methods. Mean data ± SD in parts B and D.

Intracellular Ca^{2+} release pathways were also measured using epifluorescence microscopy. The fluorochrome was excluded from lipid

droplets and thus represented cytoplasmic changes of $[Ca^{2+}]$. Figure 2.7 shows that ATP (1-100 mM) generated a Ca^{2+} transient that persisted in Ca^{2+}-free superfusate, which suggests that Ca^{2+} release was from intracellular stores. The mobilization of stored Ca^{2+} from the endoplasmic reticulum (ER) through inositol 1,4,5-triphosphate (IP3) or ryanodine receptors (RyR) was also characterised. In adipocytes the Fluo-4 dye was excluded from the lipid droplets. Within a few seconds after the addition ATP-induced waves were observed (Figure 2.7). There was considerable heterogeneity in the nature of the $[Ca^{2+}]_i$ signal observed, with some cells being less responsive, some demonstrating oscillations and waves. Also some cells demonstrated multiple $[Ca^{2+}]_i$ waves emanating from different points of the cell. Thapsigargin, an inhibitor of the ER Ca^{2+}-ATPase in Ca^{2+}-free media, initially slowed recovery from ATP evoked $[Ca^{2+}]_i$ responses and eventually abolished them, strongly suggesting that the signal arises through mobilisation of ER Ca^{2+}. ATP treatment induced elevation in intracellular $[Ca^{2+}]_i$, but did not alter IL-6 production. Caffeine, an agonist of RyRs, also caused no change in IL-6 release from adipocytes, but arachidonic acid, an IP3R inhibitor, induced IL-6 release. Treatment of adipocytes with IL-1β had no effect on the $[Ca^{2+}]_i$ responses. IL-6 release from 3T3-L1 adipocytes was measured in the culture supernatant before and after the addition of 100 μM $NiCl_2$ and 1.0 and 10.0 mM ATP. However, neither of the two agents affected the rate of release. Treatment of 3T3-L1 and isolated primary adipocytes for four hours with the cell permeable analogue of cAMP, dibutyryl cAMP (10 mM) significantly increased IL-6 secretion. Control 18.5{15.7-35.5}; dbcAMP 134.7{71.1-171.2} pg.ml-1 $p<0.001$).

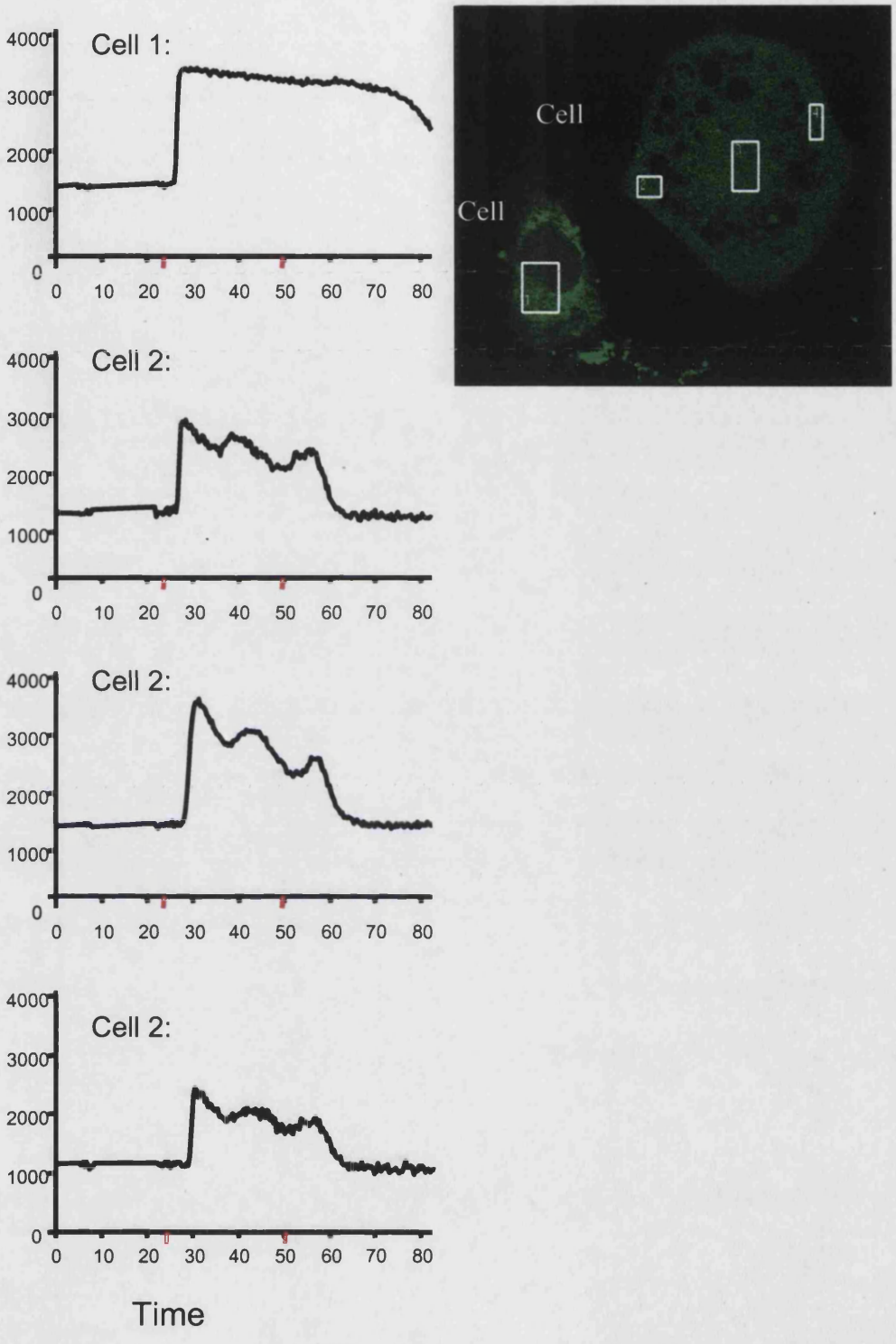


Figure 2.6F: Changes in intracellular Ca in ATP treated 3T3L1 adipocytes
These figures are a representation of changes in intracellular calcium in two different adipocytes following ATP treatment. Cell 1 RO (region of interest) shows a sustained elevation in intracellular Ca while Cell 2 shows calcium waves.

2.4.5 EP receptor agonists and IL-6 release

The results thus far suggest that cAMP, but not Ca2+-dependent pathways mediate IL-6 release from adipocytes. Therefore EP receptors utilising cAMP as a second messenger should regulate cytokine production. This hypothesis was tested by measuring IL-6 release in the presence of EP receptor agonists that raise intracellular [Ca^{2+}], such as the EP 1/3 agonist sulprostone, or those that raise cellular cAMP, such as the EP2 agonist butaprost, or the mixed EP2/3/4 agonist PGE1 alcohol. Figure 2.8 shows that sulprostone (1 μM) had no significant effect on IL-6 release. However both butaprost and PGE1 alcohol (1 μM) significantly augmented release.

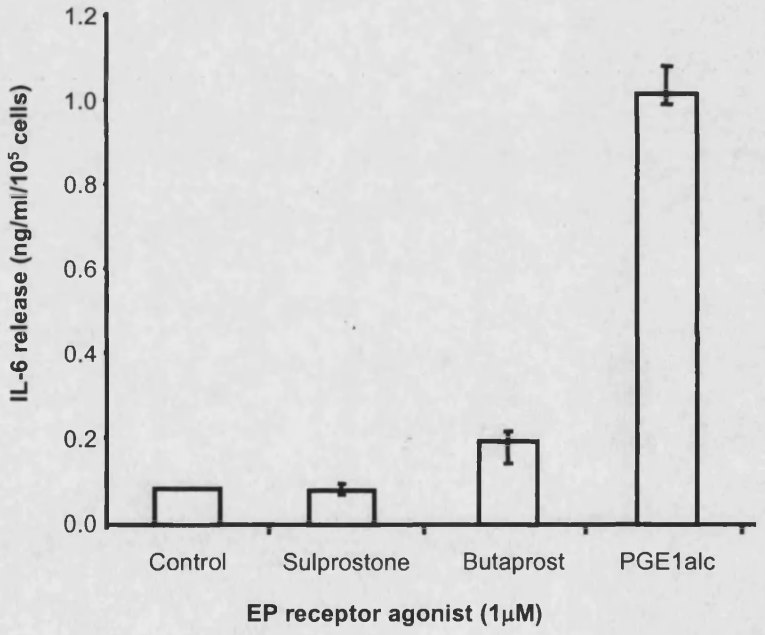


Figure 2.8: Effect of EP receptor agonists on IL-6 release in differentiated 3T3-L1 adipocytes

There was no significant change in IL-6 release from differentiated 3T3L1 adipocytes in response to Sulprostone (EP 1/3 agonist; 1μM). However both Butaprost (EP2 agonist; 1μM) and specifically PGE1 alcohol (PGE1 alcohol: EP2/3/4 agonist; 1μM) significantly augmented release.

2.5 DISCUSSION

Obesity is associated with elevated circulating interleukin-6 (IL-6) concentrations, derived from adipose tissue, a proportion of which is constitutive. This study aimed to investigate the effect of the cyclo-oxygenase (COX) pathway on basal IL-6 production from adipocytes and the molecular mechanisms involved in this secretion. In adipose tissue explants there was basal expression of COX-2 and a positive correlation between prostaglandin E2 (PGE2) and IL-6 levels in the surrounding medium. Aspirin (5 mM) and the selective COX-2 inhibitor, NS-398 ($\ll$1 μM) attenuated IL–6 release. In 3T3-L1 adipocytes PGE2 increased IL-6 secretion. The cellular pathways underlying IL-6 secretion were investigated. Ni-sensitive T-type Ca^{2+} channels were demonstrated in 3T3-L1 cells and intracellular Ca^{2+} release via ryanodine receptors was generated by ATP. However, neither $NiCl_2$ nor ATP had any effect on IL-6 production.

Prostaglandins signal via EP receptors (EPs). EP-1 activation signals through a rise of the intracellular [Ca^{2+}], but the EP-1 agonist, sulprostone, did not alter IL-6 release. However, the mixed EP-2/4 agonist, prostaglandin-E1 alcohol, and to a lesser extent the EP-2 agonist butaprost, which raise intracellular cAMP, significantly augmented IL-6 release, as did the membrane permeable cAMP analogue, dibutyryl cAMP (10 mM). Basal IL-6 release occurred through a golgi-dependant process which was transcriptionally regulated, but IL-6 was not stored in adipocytes prior to release. Thus, basal adipose tissue IL-6 secretion is mediated by COX-2 mediated PGE2 generation. Release is independent of changes to intracellular [Ca^{2+}], but depends on cAMP and is mediated by EP4 receptors.

Previous data that IL-1β stimulates the production of IL-6 in 3T3L1 adipocytes were confirmed. Simultaneously, IL-6 production is induced by the production of PGE_2. It has been shown that there is a dose dependent effect of IL1β on IL-6 secretion in adipocytes. IL1β increased COX2 expression in 3T3L1 adipocytes. Similarly, PGE2 increased IL-6 secretion in adipocytes in a dose-dependent and time-dependant manner.

It was shown constitutive COX-2 protein expression in sub-cutaneous and intra-abdominal adipose tissue explants that increased over 48h incubation, (peak at 24h). Basal IL-6 secretion mimics the COX-2 expression and correlates with endogenous PGE2 production. Furthermore, COX inhibition significantly reduced IL-6 production from adipose tissue, as well as the IL-1β induced IL-6 secretion in differentiated 3T3-L1 adipocytes.

In summary PGE_2 stimulates IL-6 synthesis in 3T3.L1 adipocytes. Exogenous PGE2 and EP agonists induce IL-6 via EP4 receptors (present in AT- SV & macrophages) and elevation in intracellular cAMP.

Understanding the specific pathway of adipose IL-6 release would allow targeted modulation of this function.

CHAPTER 3: ADIPOGENESIS

3.1 INTRODUCTION

Adipogenesis, or the conversion of preadipocytes to adipocytes, has been one of the most intensely studied models of cellular differentiation (Rosen and Spiegelman 2000). In this process undifferentiated, precursor, fibroblast-like cells are converted to cells with adipocyte morphology and functional capability (Napolitano, 1963). The adipose tissue plays a central role in controlling energy metabolism within the organism. The study of how fat cells develop and are controlled is germane because of their role in obesity.

The study of adipogenesis has been facilitated by the availability of reliable *in vitro* cellular models (Rosen et al. 2000). Adipocytes are recognized increasingly as major players in a variety of physiological and pathophysiological states, such as obesity and type 2 diabetes (Rosen, 2002). Research attention has shifted from evaluating fat storage mechanisms to studying the creation and proliferation of adipose tissue in the body. Understanding the process by which cells differentiate into fat and the mechanisms involved in this pathway may hold greater promise for controlling and eliminating obesity and its co morbidities (Gregoire et al, 1998)

Experiments using *in vitro* murine models of adipogenesis, which include the 3T3-L1 and 3T3-F442A lines, have illustrated the transcriptional cascade that promotes fat cell differentiation (Rosen et al. 2000). Though major advances in the understanding of adipogenesis have been made, the details are still not entirely elucidated. Recent studies suggest that some of disorders related to obesity may be linked to a breakdown in the regulatory mechanisms that control the expression of metabolic genes in mature adipocytes. Some progress toward an understanding of these processes has come from studies

involving the identification of transcription factors that regulate adipogenesis (Rosen et al, 2000).

3.1.1 Adipogenic transcription factors

Differentiation of adipogenic precursor cells into mature adipocytes is a complex phenomenon. It follows a highly ordered sequence (Fajas, 2003). One of the first steps in the process of adipogenesis is the re-entry of growth-arrested preadipocytes into the cell cycle and the completion of several rounds of clonal expansion.

Many aspects of adipogenesis can be described as a cascade of gene expression characterized by an ordered expression of adipocyte-specific genes, triggered by a set of interacting transcription factors (Rosen et al. 2000). The most important transcription factors involved in this process are the γ forms of peroxisome proliferator activated receptors (PPAR γ1 and γ2) and the various members of the CCAAT enhancer binding proteins (C/EBP) (Wu et al, 1995; Umek et al, 1991). PPARγ2 and C/EBPα are major controllers in the process of fat cell development and growth (McNeel and Mersmann 2000). They regulate other transcripts that produce proteins characteristic of mature fat cells. PPARγ2 expression is limited to adipose tissue where it plays a central role in committing multipotential stem cells to the adipogenic lineage, as well as regulating differentiation of preadipocytes into adipocytes (Tontonoz et al, 1994).

3.1.2 COX pathway and its metabolites in adipogenesis.

There is evidence that the COX pathway might be involved in regulating body fat and adipogenesis (Shillabeer et al, 1998). Arachidonic acid metabolites of the lipoxygenase as well as the cyclooxygenase pathway may be involved in

regulating preadipocyte differentiation. However, results from several studies are conflicting and therefore inconclusive (Wolf, 1996; Lu et al, 2004; Fain et al, 2001; Lepak and Serrero 1993). Many reports imply specific roles for prostanoids produced by the mature adipocytes in the maintenance of terminal differentiation through an autocrine/paracrine control mechanism. Some of them, such as PGJ2, serve as potent natural ligands for PPARγ, an important adipocyte transcription factor (Debril et al, 2001; Miwa et al, 2004; Nosjean et al, 2002). In 3T3-L1s it has been shown that the expression of COX-2 was induced transiently in a biphasic manner upon the triggering of the differentiation and maturation phases while COX-1 was constitutive (Lu et al, 2004; Yan et al, 2003). It has also been confirmed that PGD2 is formed from arachidonic acid by the mature adipocytes, suggesting conversion into PGJ2 derivatives (Lu et al, 2004). Inhibition of either COX-1 or COX-2 has been shown to facilitate differentiation, suggesting that both COX inhibit differentiation (Yan et al, 2003; Peterson et al, 2003). However there are other data showing opposite effects. Nishimura et al. demonstrated COX-1 inhibition suppressed adipogenesis, suggesting a role of constitutive COX-1 in the endogenous synthesis of PGs and their ability to promote adipogenesis (Nishimura et al, 2004).

3.2 Aims(s)

Having established the importance of the COX pathway in the regulation of secretion of adipose IL-6 in the preceding chapter, effects of inhibitors of these enzymes on adipogenesis were investigated.

These studies were undertaken to examine the potential role of COX pathway in modulating adipogenesis and to dissect the relative contributions of the two isoenzymes in this process.

To evaluate the role of COX pathway in the adipogenic changes, the effects of aspirin as a nonselective COX inhibitor and SC-560 and NS-398 as selective inhibitors of COX1 and COX 2 were investigated.

As cells are committed to the adipogenic cycle early effects were investigated during the first 72h (3-day) or 120h (5-day) in the presence or absence of various COX inhibitors.

3.3 METHODS

3.3.1 Preadipocyte Cell Culture and adipocyte differentiation

3T3-L1 preadipocytes were grown on 75 cm^2 tissue culture flasks with 10 ml of Dulbecco's Modified Eagle Medium (DMEM) containing 1% penicillin/streptomycin plus 10% bovine calf serum (BCS), at 37°C and 10% CO_2. When cells were nearly confluent (approximately 85%) they were trypsinised and seeded for differentiation or expanded.

The cells were seeded onto 25 cm^2 flasks or 6-well tissue culture plates at a density of $4x10^5$ cells/4ml media/flask or $6x10^4$ cells /1.5 ml media/well, and incubated at 37°C and 10% CO_2 DMEM/BCS. After 24h the media was replaced with fresh media, DMEM containing 1% penicillin/streptomycin and 10% cosmic calf serum (CCS). At confluence, preadipocytes were treated for 72 hours, at 37°C and 10% CO_2, with a differentiation induction media consisting of DMEM with 10% CCS supplemented with the glucocorticoid, dexamethasone (0.1 μM), the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX, 0.5 mM), insulin (5 μg/ml) and the

thiazolidinedione BRL 49653 (rosiglitazone, 1 μM). Induction media caused growth arrest and entry into the adipogenic cell cycle, followed by expression of adipocyte genes and lipid accumulation. Following 72h of induction the dexamethasone, IBMX and BRL 49653 were withdrawn and the cells fed with fresh DMEM/CCS containing 1 μM insulin (4 ml/flask or 1.5 ml/well). Cells were maintained in this media, which was replaced every 48 hours, until more than 90% of them were lipid containing adipocytes.

3.3.2 Interventions

The following COX inhibitors were used in these experiments: Vioxx or NS-398, COX-2 selective inhibitor at concentrations of 0,0.01,0.1 and 1.0μM, piroxicam or SC-560, COX-1 selective inhibitors at 0,0.01,0.1 and 1.0mM or 0,0.01,0.1 and 1.0μM respectively, aspirin, a non-selective COX inhibitor at 0.2, 1.0, 2.0, 5.0 mM. NS-398 and SC-560 were obtained from Cayman, Chemical, Vioxx was obtained from Merk Sharp & Dohme and aspirin and piroxicam were obtained from Sigma, Chemical.

In two different sets of experiments, various doses of aspirin (0.2, 1.0, 2.0, 5.0 mM) were added from day 0 (day of differentiation) to day 3 in one group and from day 0 to until day 5 in the other. Media were removed and retained for assay, and the 25 cm^2 flasks or 6-well plates were flash frozen in liquid nitrogen and stored at –80°C prior to RNA isolation.

3.3.3 Microscopic examination

The cells were examined by light microscopy (Nikon, Eclipse) when media was changed.

A subset of cells were differentiated, on cover slips, in the absence or presence of aspirin, and used to assess lipid accumulation by confocal

microscopy. Cells were loaded with 40 μM Bodipy 493/503, a neutral lipid dye, in HEPES buffer pH 7.4, for 15 minutes and viewed with a Zeiss confocal microscope. Images were processed using LSM 5 Image Processor.

3.3.4 RNA isolation and cDNA synthesis

RNA was isolated from cultured adipocytes as it was described in chapter 2 (page 58).

Differentiated adipocytes were digested in 1 ml TRI-reagent (Sigma) and homogenised by pipetting. Total RNA was isolated in chloroform, precipitated overnight at 4°C in isopropyl alcohol, quantified by $OD_{260/280}$ and visualised on a 1% agarose gel containing 5% formaldehyde in MOPS buffer.

cDNA was synthesized from the RNA samples by mixing 0.5μg of the isolated RNA, 7 μl 5x reverse transcriptase buffer, 3.5 μl 0.1M DTT, 2 μl 20 mM dNTP, 1 μl 40 U/μl RNAse inhibitor and 0.5 μl 50 pmol random oligonucleotide primers and Rnase free water up to a total volume of 33μl (Promega, Southampton, UK).

3.3.5 Taq-man Real-time PCR Analysis

Taq-man Real-time PCR was performed as it has been explained in chapter 2 in details (page 59).

25 μl total volume of samples (consisted of 1.25 μl specific primer, 12.5 μl SYBR-Green solution, 1 μl cDNA and 10.25 μl nuclease-free water) per well was run in duplicate. A negative control for each primer was included in PCR reaction. GAPDH as a control mRNA was also run in duplicate.

Data were obtained from a minimum of at least six separate experiments.

GAPDH, PPARγ, C/EBPα, and adipsin were used in this set of experiments.

3.4 RESULTS

3.4.1 Morphological examination by light microscopy

In control cells, after 72 hours of induction of differentiation most of the cells (>90%) had changed from spindle shaped fibroblast-like preadipocytes to the rounded morphology of the adipocyte. In this batch of cells, at days 5 to 7 after induction of differentiation, the majority of cells were differentiated and grown in volume until more than 90% of the cell was lipid.

Changes from preadipocyte to adipocyte transformation were less pronounced when using different concentrations of aspirin in a dose dependent manner. Microscopically, there was no difference between adipocyte controls (Cellgro) and those treated with 0.2mM aspirin. However, 1 and 2mM aspirin significantly reduced the number of adipocyte-like cells. At the highest dose of aspirin (5mM) none of the cells adopted an adipocyte-like morphology or accumulate lipid.

There was no difference between adipocyte controls and those treated with lower doses of Vioxx (0.01 and 0.1μM). However aspirin (1.0 or 2.0 mM), piroxicam (1mM) and SC-560 (0.01, 0.1 and 1.0 μM) reduced the number of adipocyte-like cells. Similarly, in these groups of cells there was reduced lipid content (with light microscope).

Conversely intervention with higher doses of Vioxx (1μM) resulted in larger lipid droplets within the cell when compared with untreated cells.

Confocal microscopy with the neutral lipid dye BODIPY 493/503 confirmed the observation that aspirin decreases the lipid content of adipocytes during differentiation.

3.4.2 Real time Taq-man PCR

To determine a mechanism for the effect of aspirin on adipogenesis, RT-PCR was used. Adipsin expression was investigated as an adipocyte marker that increased throughout adipogenesis.

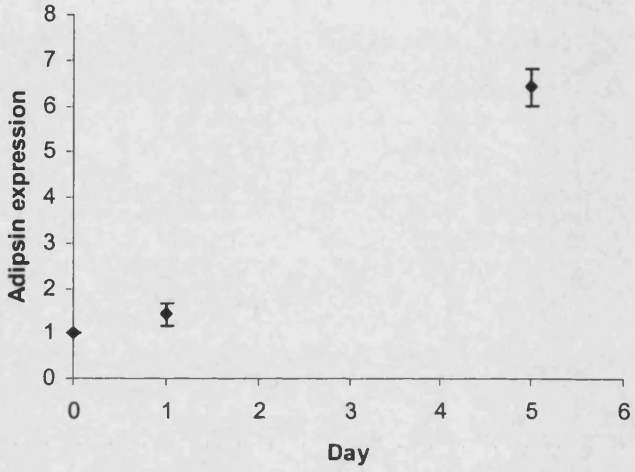


Figure 3.1: Adipsin mRNA expression in 3T3.L1 adipocytes from D0 (day of differentiation) to D5 (day 5)

Increased expression of adipsin, an early adipogenic marker, has been shown after five days post-induction.

After 5 days of induction and differentiation the aspirin treated cells had significantly lower expression of adipsin compared to those differentiated without aspirin. The inhibition was more significant with higher doses of aspirin (5mM) (Figure 3.2). Decrease in adipsin expression in aspirin treated cells confirms our microscopic examinations. Piroxicam as a selective COX 1 inhibitor appears to have no effect on adipsin expression at lower

concentrations (0.01 and 0.1mM), but 1mM of piroxicam significantly reduced adipsin expression.

Adipsin expression was increased in response to NS-398 as a selective COX 2 inhibitor (0.1μM and 1.0μM), although the rise was not statistically significant at the lower concentration (0.01μM).

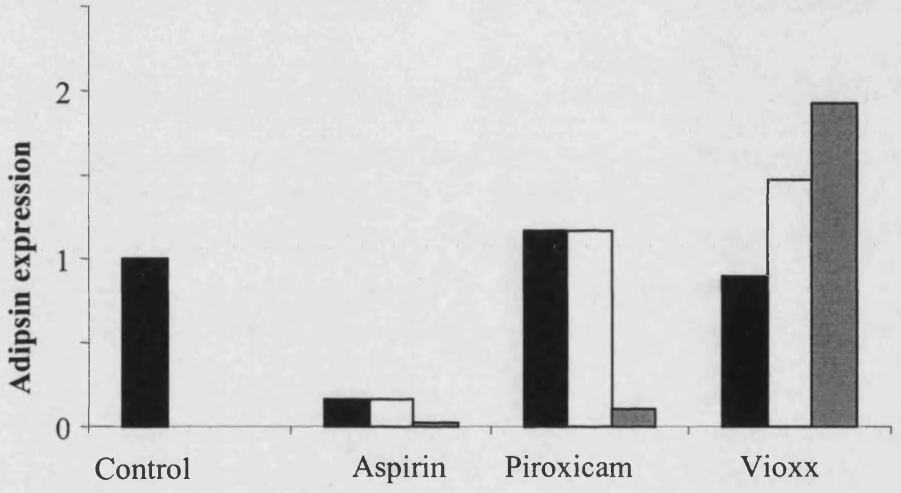


Figure 3.2: Effect of chronic exposure of COX inhibitors on adipsin mRNA expression in 3T3L1 from D0 to D5.

Significant reduction in adipsin mRNA expression has been seen in aspirin treated cells and also with higher concentration (1mM) of selective COX-1 inhibitor, piroxicam. Vioxx, a selective COX-2 inhibitor, had no effect or even positive effect on adipsin expression.

Aspirin (black: 0.2mM; white: 1.0mM; grey: 5.0mM), Piroxicam (black: 0.01mM; white: 0.1mM; grey: 1.0mM), Vioxx: (black: 0.01μM; white: 0.1μM; grey: 1.0μM)

To investigate a possible mechanism of aspirin on adipocyte differentiation, the expression of a number of adipocyte differentiation-related genes were examined. PPARγ and C/EBPα are essential for adipocyte differentiation. Expression of both is maintained at a low level in preadipocytes, but is quickly up-regulated following induction of differentiation. Increasing PPARγ and C/EBPα expression in control cells was demonstrated from the preadipocyte state to 5 days post-induction in (Fig 3.3 and 3.4)

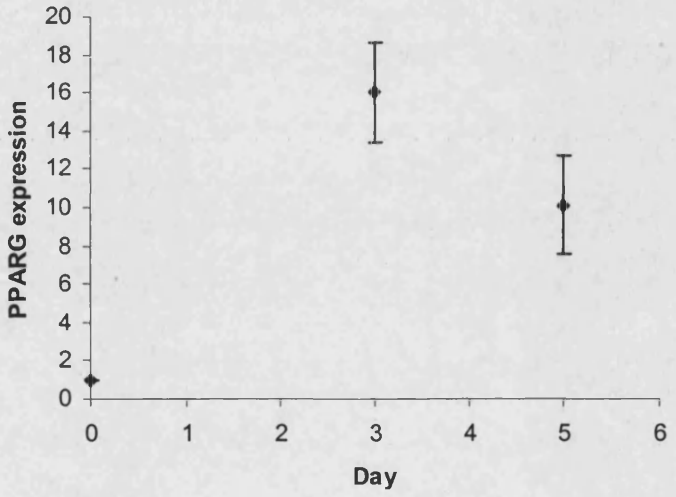


Figure 3.3: PPARγ expression in 3T3.L1 adipocytes from D0 (day of differentiation) to D5 (day 5)

Increased mRNA expression of PPARγ a major adipogenic transcription factor has been shown immediately following induction of adipocyte differentiation and after five days post-induction

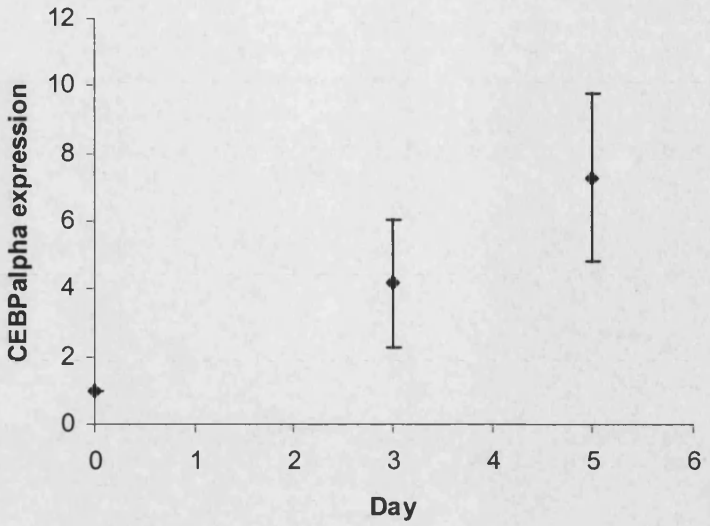


Figure 3.4: CEBPα expression in 3T3.L1 adipocytes from D0 (day of differentiation) to D5 (day 5)

Increased mRNA expression of CEBPα, an adipogenic transcription factor that along with PPARγ, facilitates adipogenesis, is apparent early in the induction of differentiation and after five days post-induction

In the cells receiving intervention with aspirin (>0.2mM) and Piroxicam (1mM) there was also inhibition of adipocyte transcription factors, PPARγ and C/EBPα, especially after exposure for 5 days.

Investigation of these two major differentiation factors shows dose-dependent down-regulation of both genes in aspirin treated cells. The trend of down-regulation of PPARγ gene in aspirin treated cells was nearly identical on day 3 and 5. However, when using 5 mM the effect was distinctly greater after 5 days.

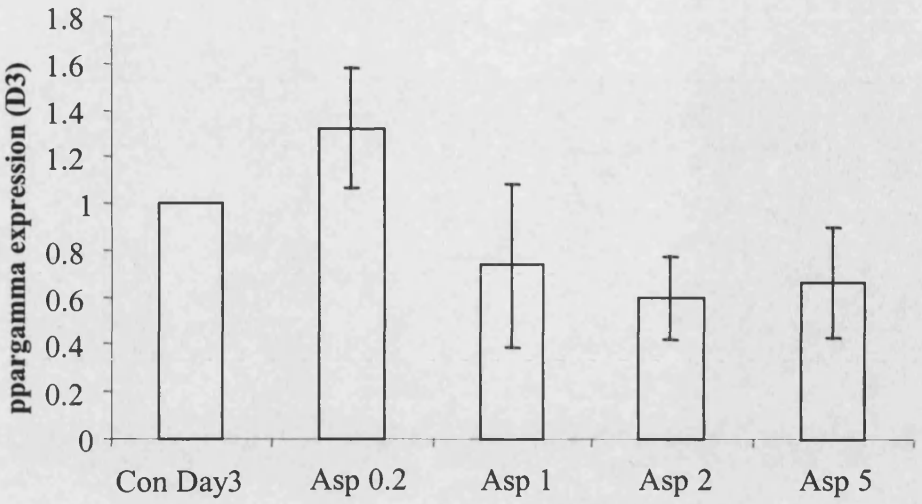


Figure 3.5: Effect of aspirin on PPARγ expression during adipogenesis of 3T3.L1 preadipocytes Figure shows significant dose-dependent inhibition of PPARγ expression as an adipocyte transcription factor at the mRNA level after treatment with aspirin for 3 days. (Con, Control; Asp0.2, Aspirin 0.2mM; Asp1, Aspirin 1mM; Asp2, Aspirin 2mM; Asp5, Aspirin 5mM)

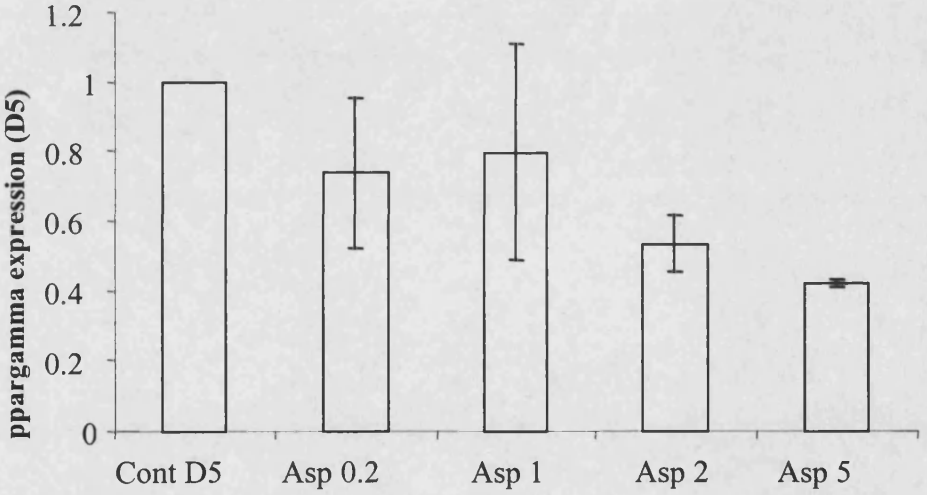


Figure 3.6: Effect of aspirin on PPARγ expression in 3T3.L1 from the preadipocyte state to 5 days post-induction

Figure shows significant inhibition of PPARγ expression as an adipocyte transcription factor at mRNA level after treatment with aspirin for 5 days. (ConD5, Control day 5; Asp0.2, Aspirin 0.2mM; Asp1, Aspirin 1mM; Asp2, Aspirin 2mM; Asp5, Aspirin 5mM)

Comparison of the expression of C/EBPα, in cells treated with or without different concentrations of aspirin during differentiation, indicated down-regulation in a dose-dependent manner after 72 hours. The trend of inhibition was the same after 5 days despite a non-significant increase in C/EBPα expression with 1 and 2 mM aspirin compared to 0.2 mM. But obviously, C/EBPα expression was affected with higher concentration of aspirin (5mM) during differentiation as well as 5 days post-induction.

In conclusion, the primary observation, (inhibition of differentiation), was confirmed using adipsin as a marker of adipocyte differentiation and transcription factors as markers of adipogenesis.

Aspirin suppressed adipogenesis in a dose-dependent manner. Higher concentrations of SC-560 and piroxicam, (as specific COX-1 inhibitors), also suppressed adipogenesis. NS-398, a selective COX-2 inhibitor, however, induced adipogenesis, and dose-dependently increased cellular lipid accumulation and induced greater number of preadipocyte to adipocyte conversion.

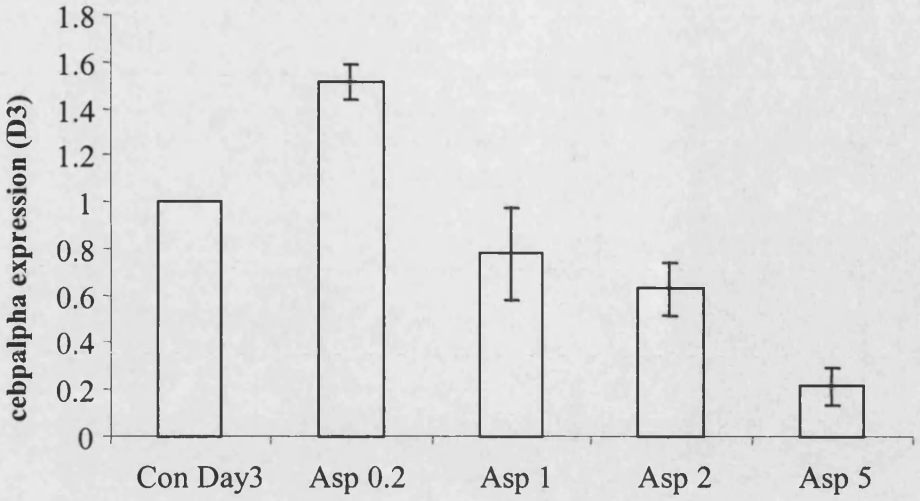


Figure 3.7: Effect of aspirin on CEBPα expression in 3T3.L1 from the preadipocyte state to 3 days post-induction Figure shows significant inhibition of CEBPα expression as an adipocyte transcription factor at mRNA level after treatment with aspirin for 3 days. (Con, Control; Asp0.2, Aspirin 0.2mM; Asp1, Aspirin 1mM; Asp2, Aspirin 2mM; Asp5, Aspirin 5mM)

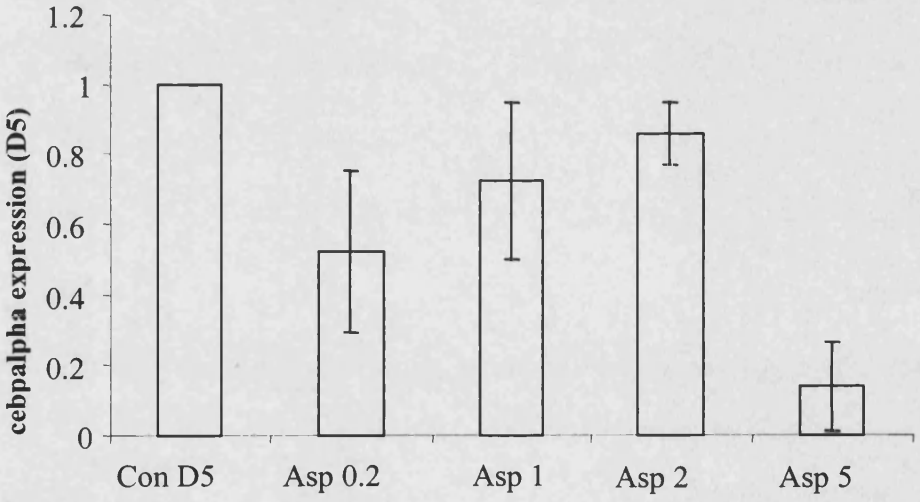


Figure 3.8: Effect of aspirin on CEBPα in 3T3.L1 from the preadipocyte state to 5 days post-induction Figure shows significant inhibition of CEBPα expression as an adipocyte transcription factor at mRNA level after treatment with aspirin for 5 days. (ConD5, Control day 5; Asp0.2, Aspirin 0.2mM; Asp1, Aspirin 1mM; Asp2, Aspirin 2mM; Asp5, Aspirin 5mM)

3.5 DISCUSSION

It is well accepted that there are two isoforms of cyclooxygenases, COX-1 and COX-2. COX-1 is expressed constitutively in most tissues and appears to be responsible for various physiological functions. COX-2, which is a key enzyme in prostaglandin synthesis, is an immediate-early response gene that is rapidly induced by many factors such as growth factors and cytokines.

To investigate a possible mechanism of aspirin on adipocyte differentiation, morphological changes, as well as alterations in the expression of a number of adipocyte specific- and differentiation-related genes were examined. Central to the mechanism of adipocyte differentiation are the transcription factors C/EBPα and PPARγ (Brun et al, 1997; Rosen, 2002), which control the expression of numerous genes required for adipose conversion and function, and are essential for adipose conversion to occur. Over-expression of either PPARγ (Tontonoz et al, 1994) or C/EBPα (Freytag et al, 1994; Wu et al, 1995) leads to differentiation. Alternatively, incubation of preadipocytes with a PPARγ ligand, such as the thiazolidinediones, is sufficient to cause adipocyte differentiation (De Vos, 1996; Mizukami et al, 1997).

Chronic exposure of differentiating adipocytes to aspirin inhibited the expression of adipogenic transcription factor PPARγ. Aspirin also inhibited C/EBPα, the second main transcription factor of adipocyte differentiation. These reductions would negatively affect differentiation and therefore adipogenesis. The down-stream effect of PPARγ inhibition would be the abrogation of various PPAR-regulated genes. Among them are the genes that are required for adipogenesis and also the ones that are important in the function of mature adipocytes. Aspirin reduced adipsin expression, as a

marker of adipocyte differentiation. The reduction could be the consequence of PPARγ and C/EBPα inhibition. This observation confirmed data, obtained from microscopic evaluation, of maintenance of pre-adipocyte-like morphology.

Aspirin suppressed adipogenesis in a dose-dependent manner. Higher concentrations of SC-560 (0.1 and 1 μM) and piroxicam (1mM) as specific COX-1 inhibitors, also suppressed adipogenesis. NS-398, a selective COX-2 inhibitor, not only had negative influence on the maturation processes, but also increased fat lipid droplets and mature adipocytes dose-dependently.

Previous data showed that (chapter 2), aspirin as a non selective COX inhibitor and Vioxx as a selective COX-2 inhibitor decrease IL-6 release from adipocytes. In spite of some data that showed IL-6 could induce PPARγ activation and therefore adipogenesis, it is unlikely that the effect of these COX inhibitors on adipogenesis could be through inhibition of IL-6 release.

First of all, the direct mechanism of IL-6 on differentiation has yet to be elucidated and according to the data in our group it has an effect on lipid engorgement compared to adipogenesis. Secondly, as it has already been shown, both aspirin and Vioxx have inhibitory effects on IL-6 release, but in terms of adipogenesis they behave differently. Another important factor which should be considered is the role of prostaglandin in differentiation. Prostaglandins are major regulators of cell growth, differentiation, and homeostasis. They are made through the action of COX pathway. Cyclooxygenase (COX) catalyzes the rate-limiting step of prostanoid biosynthesis and controls the first committed step of prostanoid formation. The availability of this enzyme directly relates to the abundance of prostaglandin

production. But the patterns of COX-1 and COX-2 expressions are distinctly different. They might therefore play different roles in adipose cell biology. The divergent function of these two isoforms may be attributable to the availability of the enzyme under particular settings or the functional coupling with different downstream terminal PG synthases.

CHAPTER 4: ADMA/DDAH AXIS IN ADIPOSE TISSUE

4.1 INTRODUCTION

Obesity is closely associated with the development of type 2 diabetes and cardiovascular disease (Pi-Sunyer et al, 2002; Caballero et al, 2003). Both insulin sensitivity and endothelial nitric oxide (NO) bioavailability are modulated by adiposity and altered NO levels may explain the insulin resistance and endothelial dysfunction of obesity (Ziccardi et al, 2002; Hamdy et al, 2003). Although it has been postulated that adipose tissue-derived mediators act on the endothelium to produce detrimental effects, none has clearly been identified (Mohamed-Ali et al, 1998; Bergman et al, 1998).

Recently, elevated plasma concentration of the naturally occurring NO synthase inhibitor, asymmetric dimethylarginine (ADMA), has been identified as a risk factor for type 2 diabetes and cardiovascular disease (Vallance et al, 1992; Boger et al, 2003) and the circulating concentration of ADMA correlates closely with the degree of insulin resistance (Stuhlinger et al, 2002). ADMA and the inert isomer symmetric dimthethylarginine (SDMA) are released during the hydrolysis of proteins that contain arginine residues methylated by protein arginine methyltransferases (PRMTs) (Clarke, 1993). The primary route of catabolism of ADMA is by the enzyme DDAH to form citrulline and dimethlyamine (Leiper and Vallance 2003). In contrast, SDMA is excreted unchanged in the urine. Two isoforms of DDAH have been identified and expression reported in several tissues (Leiper et al, 1999).

4.2 AIMS(s)

Given the role of DDAH in the regulation of ADMA degradation, it was postulated that adipose tissue is a source of ADMA in obesity and

interventions that alter adipose DDAH expression would be expected to modulate ADMA levels.

4.3 METHODS

4.3.1 Mouse studies.

DDAH1$^{+/-}$ mice on a mixed agouti C57Bl/6 background were generated. Genotyping of mice was performed by Southern blot analysis. C57BL/6 and ob$^{-/-}$ (Harlan, UK) male, 12 week old mice were bled by cardiac puncture and then killed by cervical dislocation prior to removal of the dorsal lumbar sub-cutaneous and the epididymal adipose tissue depots.

4.3.2 Human studies.

All subjects taking part gave informed written consent to these studies, which had previously been approved by the local ethics committee.

4.3.2.1 Arterio-venous difference study.

This part of study was in collaboration with Oxford Centre for Diabetes, (endocrinology and metabolism department) at university of Oxford. Arterio-venous differences were measured in 12 healthy Caucasian male volunteers (BMI 26.6{24.2-36.7kg.m^{-2}}) studied after an overnight fast. Cannulae were inserted into the superficial epigastric vein draining the subcutaneous abdominal adipose tissue (Frayn et al, 1989), into a deep antecubital vein, draining the deep forearm tissue and, to a vein on the back of hand to provide arterialised venous blood by heating the hand in a hot box at +60 C. All lines were kept patent by a slow infusion of saline. Blood samples were taken simultaneously from the different sites. Previous work has shown that venous blood from the superficial epigastric veins approximates the effluent from an

adipose tissue bed, and arterio-venous differences across abdominal tissue yields results in good agreement with those of microdialysis studies. Deep antecubital vein samples approximate the venous effluent of the skeletal muscle (Frayn et al, 1993).

4.3.2.2 Weight loss study.

This part of study was in collaboration with Franz Volhard Clinic, Medical Faculty of the Charite at Humboldt University of Berlin and Max Delbruck Centre for Molecular Medicine.

Fifteen, healthy, post-menopausal, Caucasian women participated in this study. All medication was stopped at least 7 days and hormone replacement at least four weeks before the study. The women were weight stable to 1kg during three months preceding the study. Baseline blood samples and an adipose tissue biopsy were obtained in the morning after an overnight fast.

Weight reduction was achieved by dietary counselling (reduction of energy intake by 600 kcal/day). Repeat blood samples, adipose tissue biopsies and clinical measurements were taken after 5% weight loss was achieved.

4.3.2.3 Adipose tissue biopsy.

Abdominal sub-cutaneous adipose tissue samples (1.5-3.0g) were obtained by needle biopsy under local anaesthesia from the periumbilical region.

4.3.2.4 Organ culture of adipose tissue.

Subcutaneous and epididymal adipose tissue was removed from C57BL/6, $ob^{-/-}$ and $DDAH1^{+/-}$ mice. The tissue was removed and prepared for experiment as it has been described in chapter 2 (page 55).

0.2g of adipose tissue from both depots was incubated in serum-free media (Cellgro, Hyclone, USA) for 24h at 37°C / 5% CO_2 in the absence of any additives. At the end of the incubation, media was removed and retained for ADMA assay and the tissue was snap frozen in liquid N_2 and stored at –80°C for RNA and protein extraction. In intervention study, 0.2g of fat tissue from both depots was placed in 6-well plates. Tissue fragments were incubated in Cellgro containing rosiglitasone (20μM), TNFα (20ng/ml), IL-6 (10 ng/ml), PGJ2 (20 μM) and aspirin (0.2, 1 and 2 mM). After 24 hours of incubation, the tissue was frozen in liquid N_2 and stored at –80°C along with the culture supernatant. Serum-free media was used as a basal control.

4.3.3 Assays.

Insulin concentrations were determined by radio-immunoassay (DPC Biermann, Germany) and glucose by polarography (Beckmann, Germany). Insulin resistance was calculated from fasting plasma insulin and glucose concentrations by homeostasis model assessment (HOMA). DDAH activity was assessed using a radiochemical assay for the metabolism of ^{14}C-labelled L-NMMA with a few modifications of a previously reported method (MacAllister et al, 1996). Briefly, 50μl of adipose tissue lysate was incubated at 37°C for 90 minutes with 50μl of phosphate buffered saline, containing 0.02mCi of L-[^{14}C]-NMMA and 20μM unlabelled L-NMMA. Following incubation [^{14}C]-citrulline was measured by mixing with 1ml of 50% (w/v) Dowex 50X8-400 and centrifuging for 3 minutes at 13,000rpm. 100μl of the supernatant was mixed with 5ml of scintillation fluid and the ^{14}C content measured.

4.3.3.1 ADMA Extraction

ADMA measured by HPLC with fluorescence detection. 0.2 ml of plasma was mixed with 40µl of a 10ng/µl solution of the internal standard M N-monomethyl-L-arginine (L-NMMA) and 0.760 ml of PBS. In cell culture (or organ culture), 10µl L-Homoarginine (10 mg/L) was added to certain amount of supernatant (0.5 ml) as an internal standard.

This mixture was applied to Oasis MCX solid-phase extraction columns (Waters). The columns were washed consecutively with 1.0 ml of 0.1 mol/l HCl and 1.0 ml of methanol. Basic amino acids were eluted with 1.0 ml of concentration ammonia/water/methanol (10:40:50, by volume). After evaporation of the solvent under nitrogen, the amino acids were derivatized with *o*-phthaldialdehyde reagent containing 3-mercaptopropionic acid. The derivatives were separated by isocratic reverse-phase chromatography on a Symmetry C18 column (3.9 mm×150 mm; 5 µm particle size; Waters) at a column temperature of 30°C. Potassium phosphate buffer (50 mmol/pH 6.5), containing 8.7% acetonitrile, was used as mobile phase at a flow rate of 1.1 ml/min. After elution of the last analyte, strongly retained compounds were quickly eluted by a strong solvent flush with acetonitrile. Fluorescence detection was performed at excitation and emission wavelengths of 340 and 455 nm respectively. All samples from individual patients, cell or tissue supernatant were analyzed in the same analytical series.

4.3.4 Western blot analysis

Western blot analysis has been done as it has been described in chapter 2 (page 57). Protein was isolated from approximately 200mg of the adipose tissue. The tissue was crushed and lysed in protein lysis buffer (1x PBS,

containing 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) supplemented with protease inhibitors (Complete, Boehringer Mannhein, UK). The homogenate was centrifuged at 14,000rpm for 15 minutes and the infranatent recovered. The protein content was estimated with a kit from Biorad, UK.

Twenty microgram of total protein was loaded onto gels and separated by SDS-PAGE electrophoresis. Gels were blotted onto PVDF membranes and blocked with 5% non-fat dried milk and probed with monoclonal antibodies against DDAH 1 and 2 that were raised to peptides used previously to generate polyclonal antibodies to these molecules. Secondary antibodies conjugated to horse-radish peroxidase were from Amersham Bioscience. Antigen-antibody complexes were detected by chemiluminescence with an ECL kit (Amersham) and blots exposed to high performance chemiluminescence film (Kodak).

4.3.5 RNA extraction and real-time PCR.

Total RNA was isolated from adipose tissue with an RNeasy lipid tissue mini-kit (Qiagen). Two microgram of RNA was reverse-transcribed to cDNA with the use of random hexamers (Retroscipt, Ambion, Inc.). Real-time PCR was performed with an ABI 5700 sequence detection system. Primers and probe sequences for mouse DDAH 1 and DDAH 2 were selected with Primer Express (Perkin-Elmer Applied Biosystems). Primer-probe sets for human DDAH1 and 2 (Assays-on-Demand) were from Applied Biosystems. Each gene assay was run in a singleplex reaction in duplicate with Taqman universal PCR master mix (Applied Biosystems); PCR reactions (25µl) contained 3µl of 1:100 diluted cDNA. GAPDH expression levels were used to normalize gene expression in each sample.

4.3.6 Statistical analysis.

Results are expressed as mean±SD or median (interquartile range). Comparisons between two groups were made by paired or unpaired two-tailed Student's t-test, Mann-Whitney U test or Wilcoxon rank test, as appropriate. $P< 0.05$ was considered to be statistically significant.

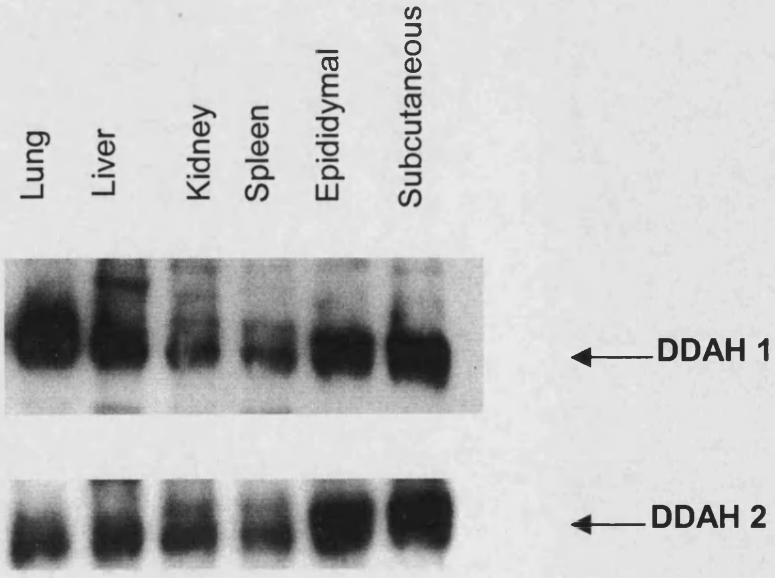
4.4 RESULTS

4.4.1 Protein expression

Expression of both DDAH isoforms was investigated in a murine preadipocyte cell-line, 3T3-L1, using western blot. Both undifferentiated and differentiated adipocytes expressed DDAH 1 and 2, however adipocytes expressed less DDAH 1 and 2 compared to the preadipocytes.

DDAH1 and 2 proteins were also investigated in sub-cutaneous and epididymal adipose tissue. Significant expression and activity was found at levels comparable to those seen in tissues such as kidney and liver (Fig 4.1).

Figure 4.1: DDAH protein expression in different tissues



On comparing the level of DDAH protein expression in adipose tissue from ob/ob mice, as a genetic model of obesity, compared to lean C57BL/6 controls, lower levels of protein expression were observed in subcutaneous adipose tissue in obese mouse (Fig 4.2).

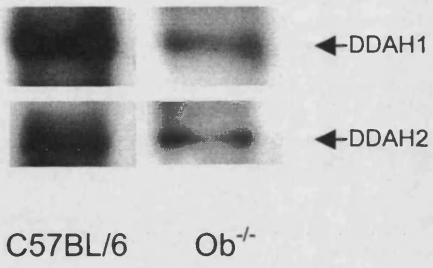


Figure 4.2: DDAH expression in subcutaneous murine adipose tissue (C57BL/6 and ob/ob)

4.4.2 mRNA expression

At the mRNA level, both preadipocytes and differentiated adipocytes expressed DDAH 1 and 2 that was confirmed by using Taq-man real time PCR. However, the expression of DDAH 1 and 2 was less in adipocytes than preadipocytes (Fig 4.3).

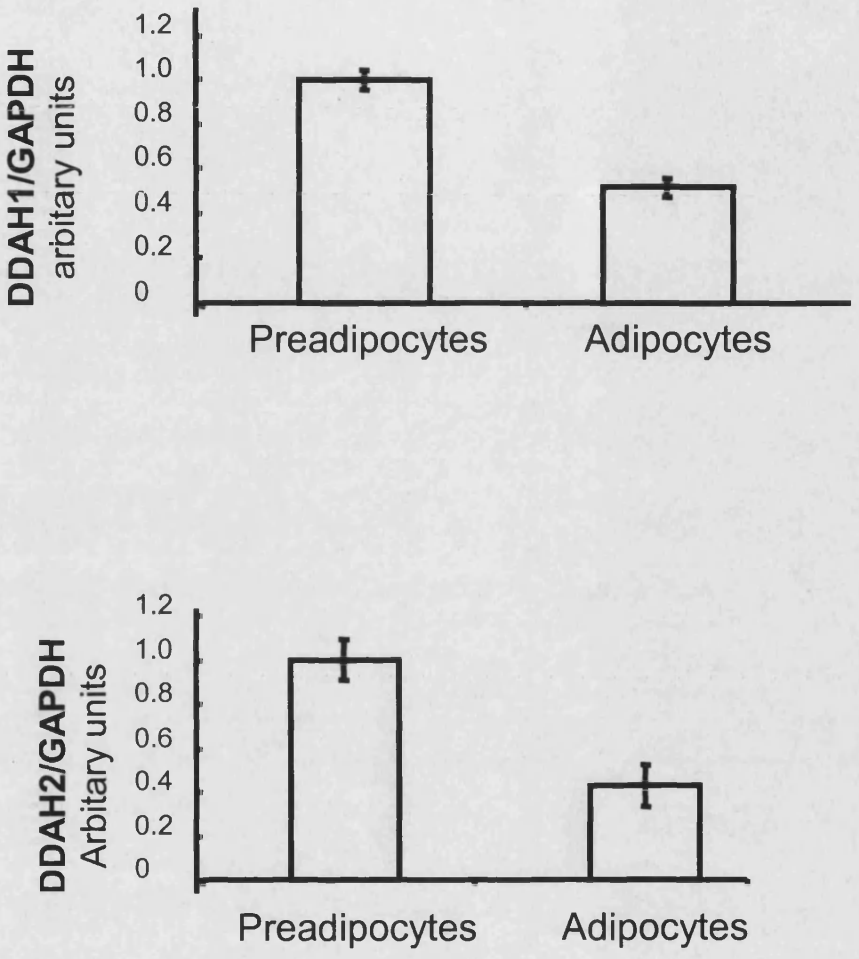


Figure 4.3: mRNA expression of DDAH 1 and 2 in preadipocytes and adipocytes by Taq-man real time PCR

DDAH mRNA expression was investigated in subcutaneous and epididymal adipose tissue from C57BL/6 and ob/ob animals. It was shown that ob/ob animals expressed less DDAH 1 and 2 compared to C57BL/6 controls (Fig 4.4).

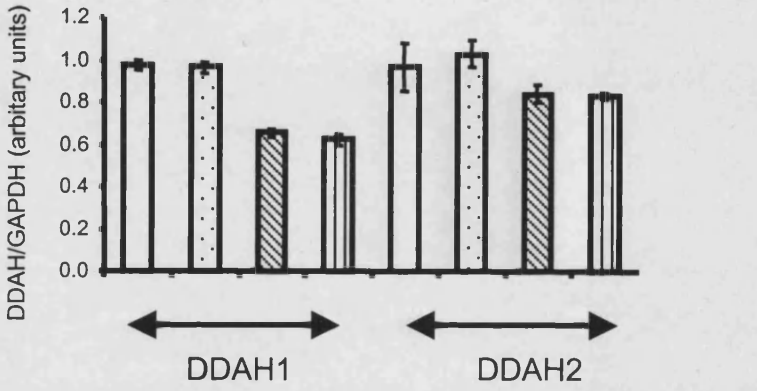


Figure 4.4: mRNA expression of DDAH 1 and 2 in murine adipose tissue (C57BL/6 versus ob/ob) by Taq-man real time PCR

In human adipose tissue, both DDAH 1 and 2 were expressed at mRNA level. The expression of these enzymes was compared from biopsy specimens from lean and obese individuals. Sub-cutaneous adipose tissue mRNA expression of DDAH 1 and 2 were lower in obese subjects compared to lean individuals (Table 4.1).

Variable	Lean	Obese
number	18	18
age [years]	56 ± 4	58 ± 5
BMI [kg/m2]	23.9 ± 2.1	35.7 ± 4.1 *
waist circumference [cm]	79 ± 7	105 ± 10 *
glucose [mmol/l]	4.9 ± 0.5	5.4 ± 0.4 *
insulin [µU/l]	2.9 ± 1.5	6.9 ± 3.5 *
HOMA index	0.7 ± 0.4	1.7 ± 0.9 *
DDAH1/GAPDH [arbitrary units]	1.64 (1.15-2.00)	0.89 * (0.73-1.54)
DDAH2/GAPDH [arbitrary units]	5.94 (4.32-8.49)	4.71 (3.32-8.44)
ADMA (µM)	0.55 (0.52-0.58)	0.56 (0.49-0.63)

Table 4.1: Effect of obesity on adipose tissue DDAH expression and circulating ADMA *: $p<0.01$

Interestingly, with the reduction in body weight there was a median increase in adipose tissue mRNA expression levels of DDAH 1 (43% increase) and DDAH 2 (24% increase) (Table 4.2).

mRNA (arbitrary units)	Before	After
DDAH1/GAPDH	0.86 (0.64-1.21)	1.23* (0.87-2.08)
DDAH2/GAPDH	4.20 (2.91-7.49)	5.22* (3.50-9.07)

Table 4.2: Taq-man real time PCR analysis of DDAH 1 and DDAH 2 expression before and after weight loss. *: $p<0.01$

4.4.3 ADMA level

Both undifferentiated and differentiated adipocytes released detectable ADMA levels into the culture media. However, the differentiated adipocytes released significantly more ADMA, compared to the preadipocytes (Fig 4.5).

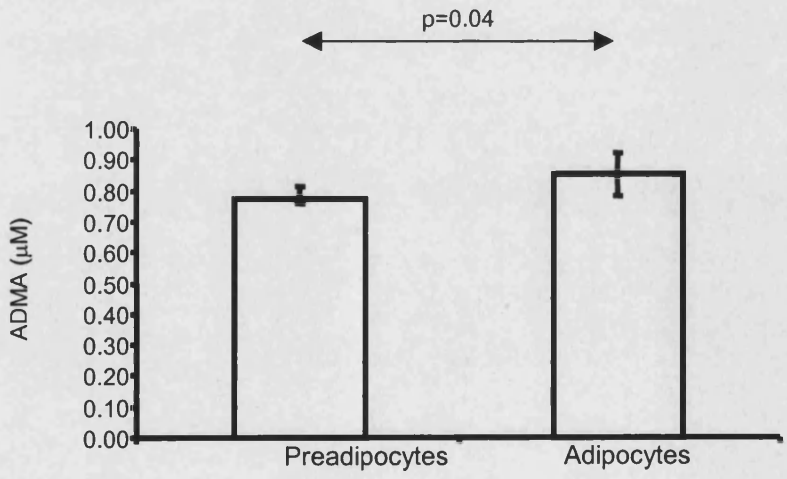


Figure 4.5: ADMA release from preadipocytes and adipocytes

In organ cultures of adipose tissue, the level of ADMA in the culture media was increased with increasing fat mass. There was also higher release of ADMA from subcutaneous fat compared to epididymal tissue (Fig 4.6). The level of adipose ADMA release from DDAH1$^{+/-}$ mice was also investigated. The release of ADMA from heterozygous explant tissue was 2.1 fold more than release of ADMA from wild-type control animals (Fig 4.6).

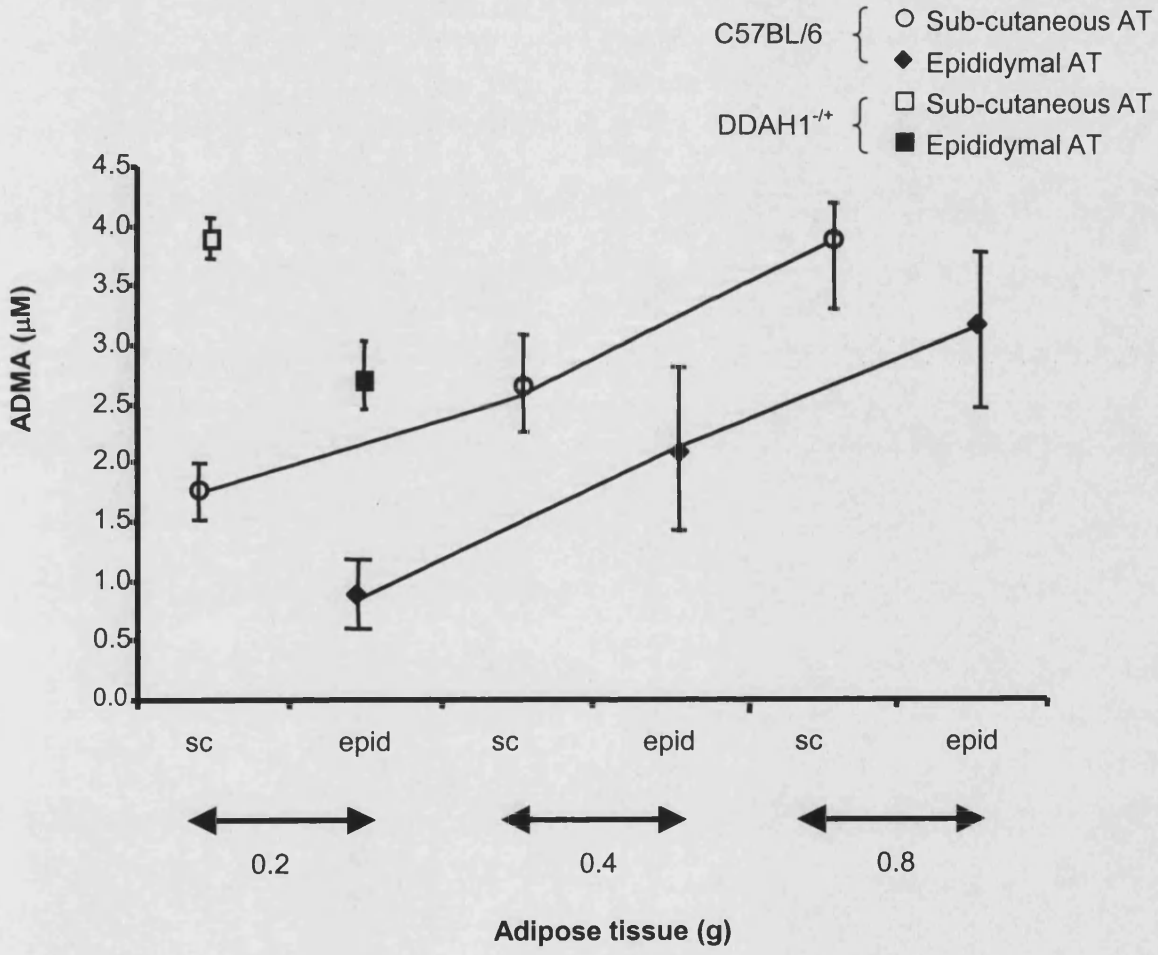


Figure 4.6: ADMA release from adipose tissue explants

ob/ob animals were used as a genetic model of murine obesity with insulin-resistance. The levels of ADMA in circulation were higher in ob/ob animals compared to lean C57BL/6 control animals (p=0.03). The release of ADMA from ob/ob explant adipose tissue was also significant higher than the release from the same amount of tissue in lean animals (Fig 4.7).

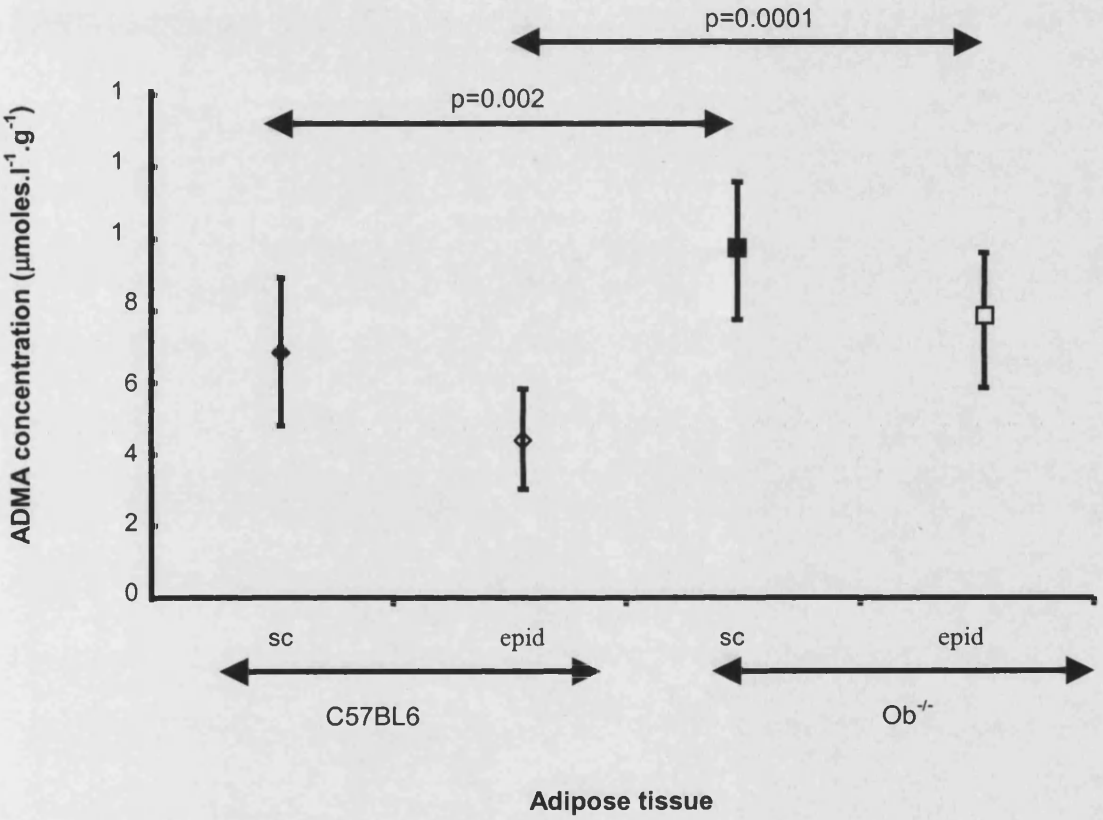


Figure 4.7: Effect of obesity on ADMA release in murine adipose tissue.

sc, sub-cutaneous adipose tissue; epid, epididymal adipose tissue, Data C57BL/6, n=8; $ob^{-/-}$, n=5. All data shown as mean ±SD.

In the human study, systemic ADMA concentrations were measured in lean and obese age-matched women. The obese subjects were hyperglycaemic and more insulin resistant compared to the lean volunteers and had

significantly higher levels of DDAH 1 mRNA expression in their adipose tissue (Table 4.1). While the trend for DDAH2 expression in the lean tissue was also higher it did not reach significance and circulating levels of ADMA was also not different between lean and obese subjects.

In the study of subjects who achieved a median weight loss of 5.2%, plasma ADMA levels after weight loss were significantly lower compared to the their levels before weight loss (Fig 4.8).

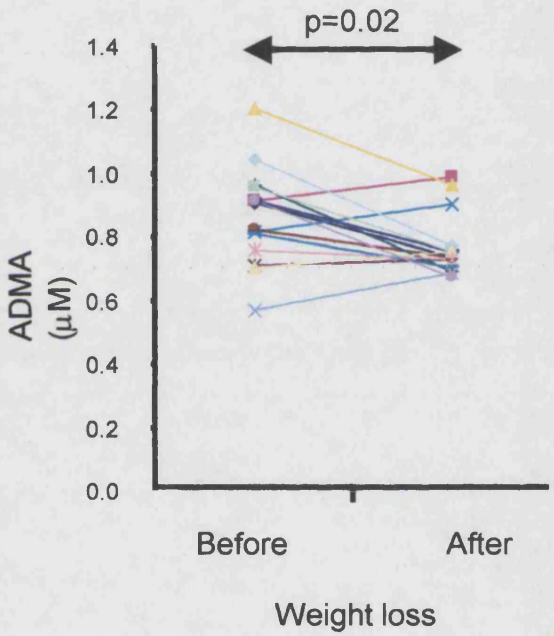


Figure 4.8: Effect of weight loss on systemic ADMA levels

Weight loss was accompanied by a significant reduction in BMI, waist circumference and HOMA index of insulin resistance. However, there was no change in plasma lipids or glucose (Table 4.3).

Variable	Baseline	After weight loss
BMI [kg/m^2]	33.1 ± 4.6	31.2 ± 4.3 *
waist circumference [cm]	101 ± 11	97 ± 11 *
total cholesterol [mmol/l]	5.7 ± 1.0	5.5 ± 1.1
HDL-cholesterol [mmol/l]	1.7 ± 0.4	1.6 ± 0.4
LDL-cholesterol [mmol/l]	3.5 ± 0.9	3.3 ± 1.0
triglycerides [mmol/l]	1.2 ± 0.5	1.3 ± 0.6
glucose [mmol/l]	5.7 ± 0.8	5.7 ± 0.8
insulin [μU/l]	4.8 ± 3.3	3.9 ± 2.5 *
HOMA index	1.2 ± 0.9	1.0 ± 0.7 *

Table 4.3: Effect of weight loss on glucose and lipid profiles *$p<0.01$

Arterio-venous differences were measured across a human abdominal sub-cutaneous adipose tissue bed and forearm muscle. ADMA levels were significantly elevated in the epigastric vein compared to that from the radial artery (Fig 4.9a). SDMA levels were not significantly different between the different sites of sampling (Fig 4.9b). Furthermore, no significant difference was detectable for ADMA across the forearm muscle in these subjects (Fig 4.9c).

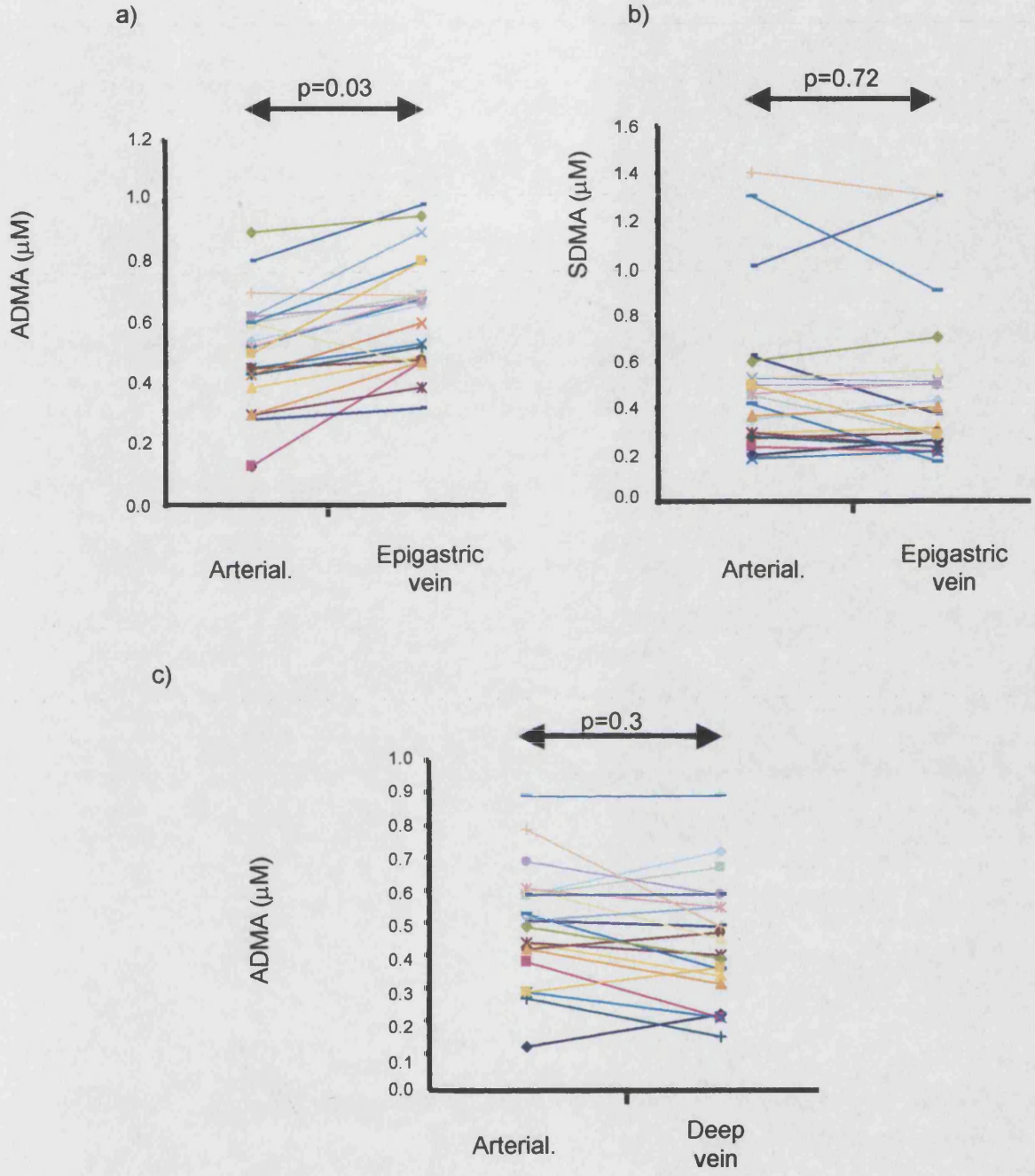


Figure 4.9: Release of ADMA by human subcutaneous adipose tissue

4.4.4 Result (Intervention study)

After 24 hours treatment of adipose tissue explants with 20μM rosiglitazone, DDAH1 expression was increased at both mRNA and protein levels (Fig 4.10a,b). There was also an increase in DDAH 2 expression, but not as much as DDAH1.

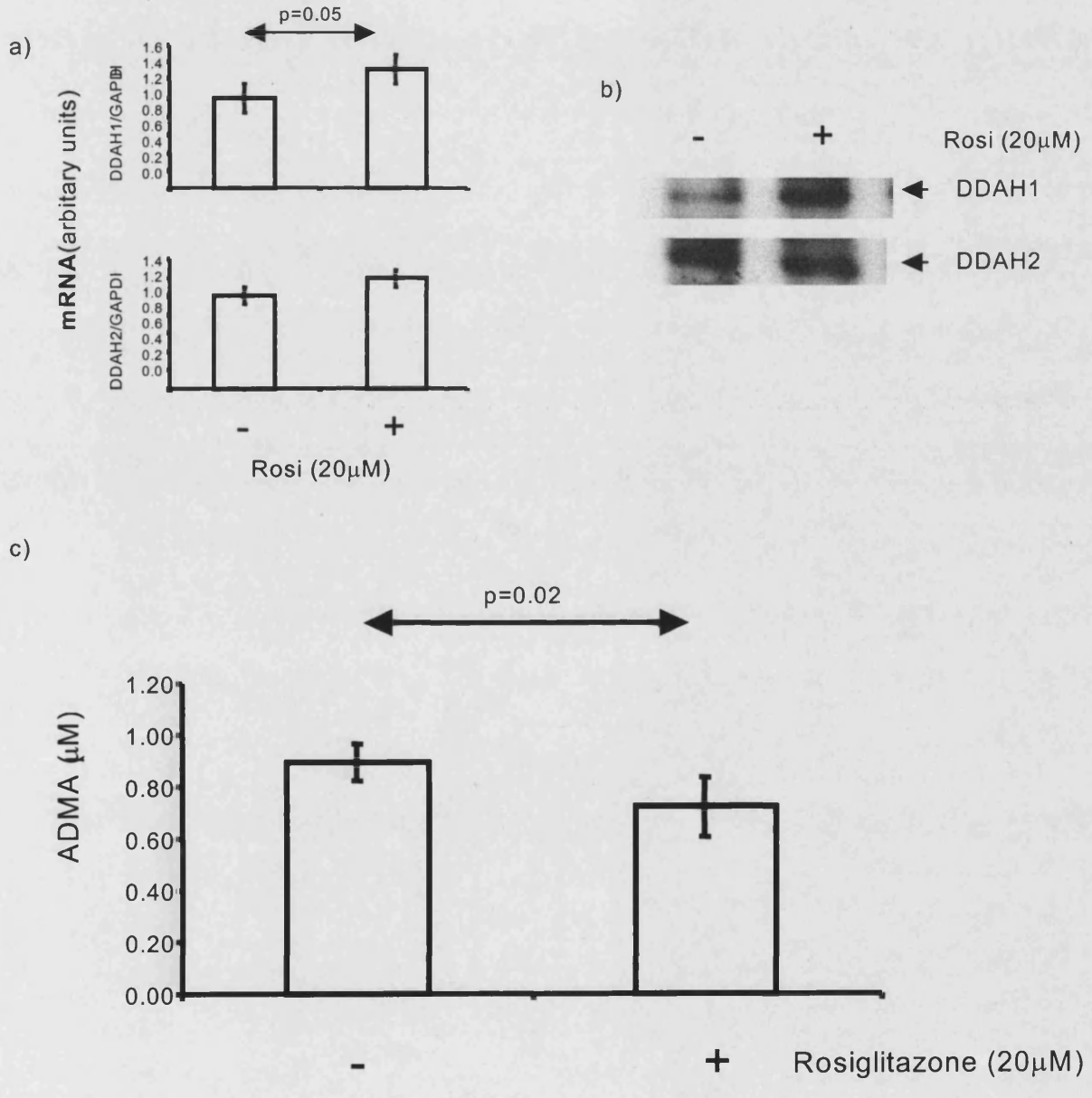
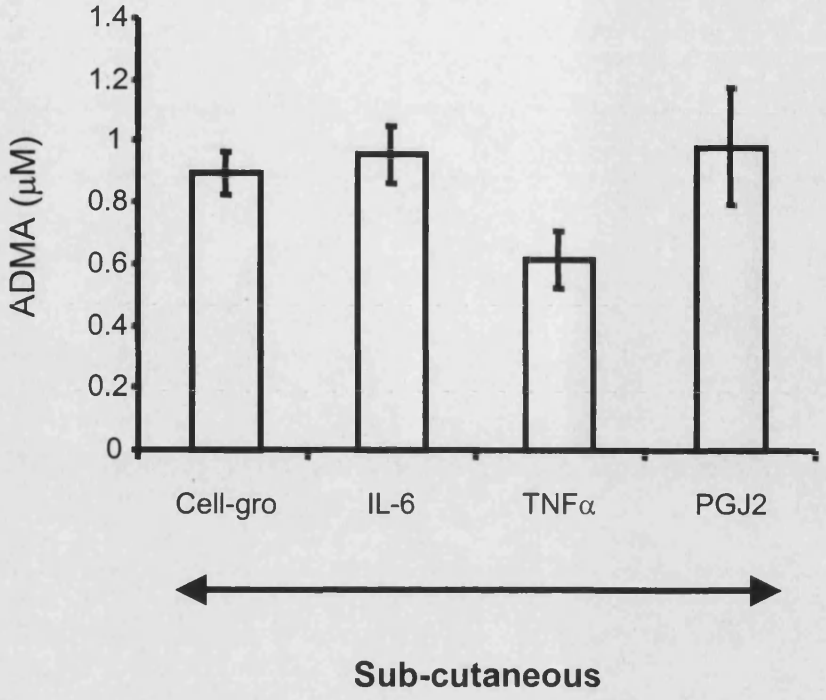


Figure 4.10: Effect of Rosiglitazone on mouse adipose tissue DDAH expression and ADMA release.

Treatment of adipose tissue explants with TNFα also increased DDAH1 protein expression, but there was not significant change in DDAH expression in IL-6 and PG J2 treated tissues. The effect of aspirin on DDAH expression was different in subcutaneous and visceral adipose tissue. In other words, they behave differently in these depots of adipose tissue, which needs more work and exploration (data has not been shown).

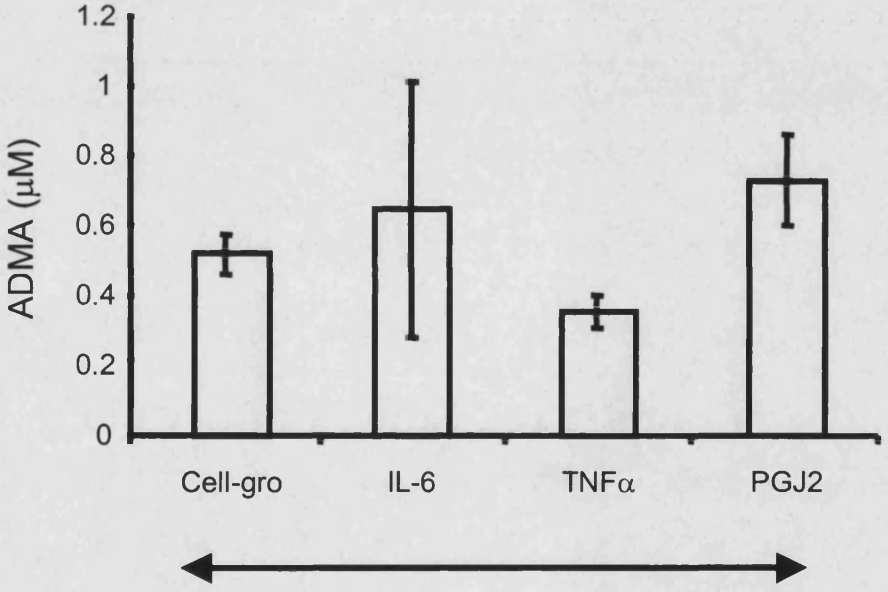
ADMA release by tissue was reduced in TZD treated explants compared to control tissues (Fig 4.10c).

TNFα (20 ng/ml) also reduced ADMA release from the subcutaneous and epididymal adipose tissue. But exposure of tissue fragments with IL-6 (10ng) and PGJ2 (20 μM) did not affect ADMA release compared to control (Fig 4.11a,b). Change in the release of ADMA in aspirin treated explants compared to basal was variable, but there was a trend of reduction of ADMA levels in subcutaneous explants compared to basal release.



Sub-cutaneous

Figure 4.11a: Effect of TNFα, IL-6 and PGJ2 on ADMA release from sub-cutaneous mouse adipose tissue.



Epididymal

Figure 4.11b: Effect of TNFα, IL-6 and PGJ2 on ADMA release from epididymal mouse adipose tissue.

TNFα (Tumor Necrosis Factor alpha: 20 ng/ml), IL-6 (Interleukin 6: 10ng) and PGJ2 (Prostaglandin J2: 20 µM); data shown as mean ±SD, n=6.

4.5 DISCUSSION

Expression of both DDAH isoforms was established in a murine preadipocyte cell-line, 3T3-L1. Both undifferentiated and differentiated adipocytes expressed DDAH 1 and 2 mRNA and released ADMA into the culture media. However, the differentiated adipocytes expressed less DDAH 1 and 2, and released significantly more ADMA, compared to the preadipocytes. DDAH1 and 2 proteins were investigated in subcutaneous and epididymal adipose tissue. Significant expression of these proteins was found at the levels comparable to those seen in kidney and liver (Fig 4.1). In organ cultures of adipose tissue, ADMA accumulation in the culture media was proportional to the mass of the tissue explants and the release of ADMA from subcutaneous fat was significantly higher than that observed from epididymal tissue (Fig 4.6). Degradation of ADMA is catalysed by DDAH and so changes in the expression of the enzyme would be expected to alter the extent of ADMA degradation by the tissue. Therefore adipose ADMA release from tissue obtained from DDAH1$^{+/-}$ mice was assessed. The explant tissue from the heterozygous mice released 2.1 fold more ADMA compared to that seen in tissue from wild-type control animals (Fig 4.6), confirming the essential role of DDAH in regulating ADMA levels within tissues.

Endothelial function is impaired in leptin-deficient ob$^{-/-}$ mice and this may be an excellent model for the study of the cardiovascular effects of obesity (Winters et al, 2000). These animals also exhibit many features of non-insulin-dependent diabetes such as mild hyperglycaemia, hyperlipidemia, and hyperinsulinemia. These insulin-resistant mice were used as a genetic model of murine obesity and found that systemic ADMA levels were higher in these

animals compared to lean C57BL/6 control animals (p=0.03). Furthermore, adipose tissue obtained from $ob^{-/-}$ mice expressed lower levels of DDAH 1 and 2, and released significantly more ADMA, compared to tissue obtained from lean animals (Fig. 4.2; 4.4; and 4.7). Thus lower overall DDAH activity in the adipose tissue of $ob^{-/-}$ animals probably accounts for the elevated ADMA secretion.

To determine whether the results observed in rodents are valid in humans, and to test the hypothesis that the adipose tissue is a significant source of ADMA *in vivo* arterio-venous differences across a human abdominal sub-cutaneous adipose tissue bed and forearm muscle were measured (Frayn et al, 1989). It was found significant elevation in ADMA levels in the epigastric vein sample compared to that in arterialized blood from a vein on the back of hand, confirming release of ADMA from this adipose depot (Fig 4.9a). SDMA levels were not significantly different between the different sites of sampling (Fig 4.9b). Furthermore, no significant difference was detectable for ADMA between the forearm deep vein and arterialized venous blood, suggesting that skeletal muscle is not a significant source of ADMA (Fig 4.9c).

Weight loss and the reduction in adipose tissue have been shown to improve insulin resistance and endothelial dysfunction (Ziccardi et al, 2002; Hamdy et al, 2003; Raitakari et al, 2004). In this study, women achieved a median weight loss of 5.2% (range 4.3 to 9.7 %) over 13 weeks (range 11 to 18 weeks) by caloric restriction. In these subjects weight loss was accompanied by a significant reduction in BMI, waist circumference and HOMA index of insulin resistance. However, there was no change in plasma lipids or glucose (Table 4.3). With the reduction in body weight there was a median increase in

adipose tissue mRNA expression levels of DDAH 1 (43% increase) and DDAH 2 (24% increase) and plasma ADMA declined significantly (Table 4.2 and Fig 4.8). Previous studies have shown that a 7-10% weight reduction improves flow-mediated vasodilatation in diabetic and non-diabetic obese individuals (Ziccardi et al, 2002; Hamdy et al, 2003; Caballero, 2004; Raitakari et al, 2004). These observations that a more modest weight loss that causes no discernable change in plasma glucose results in a fall in ADMA suggests that changes in adipose ADMA metabolism may precede detectable improvement in endothelial function in obesity.

Thiozolidinediones (TZDs) are potent insulin sensitizers that lower blood sugar (Seufert et al, 2004) but also appear to improve other abnormalities associated with type 2 diabetes, including hyperlipidemia, atherosclerosis, hypertension and chronic inflammation (Willson et al, 2001). They also ameliorate endothelial dysfunction (Dandona and Aljada 2004). In the present study treatment of adipose tissue explants with rosiglitazone (20μM), a TZD that has been shown to reduce systemic levels of ADMA (Stuhlinger et al, 2002), increased the expression of DDAH1, and to a lesser extent DDAH2, at both the mRNA and protein levels (Fig 4.10a,b). Furthermore ADMA release by the tissue was reduced in TZD treated compared to control tissue (Fig 4.10c). It has already been shown the presence of a PPARγ consensus sequence in the promoter of the DDAH2 gene (Tran et al, 2000). This data also support a role for PPARγ in the regulation of DDAH 1 expression.

Adipose tissue is partitioned into a few large depots, including the subcutaneous and visceral sites, and many small depots that lie in close proximity to a variety of organs such as muscle, nerves, blood vessels,

connective and lymphoid tissue (Pond, 1999). Obesity is characterised by enlargement of many of the adipose tissue depots as well as infiltration of insulin responsive tissues such as skeletal muscle and liver with fat (Goodpaster et al, 2002). ADMA has been purported as a fundamental link between insulin resistance, endothelial dysfunction, lipid and glucose metabolism and cardiovascular disease (Stuhlinger et al, 2002; Cooke, 2000). These data has shown that adipose tissue is a significant source of ADMA, an endogenous inhibitor of all isoforms of NOS. The amount of ADMA generated is proportional to the mass of adipose tissue and inversely related to activity of DDAH within the tissue. The ADMA generated would affect NO-mediated functions of the adipose tissue itself, such as adipocyte growth and differentiation, local blood flow, glucose uptake and lipolysis, and inhibit NO mediated pathways in other insulin sensitive organs including systemic vascular endothelium, muscle and liver. Dietary and pharmacological interventions that improve insulin sensitivity and endothelial dysfunction increased adipose tissue expression of DDAH and reduced release of ADMA, suggesting that this may be a target for future therapies for obesity associated pathologies. The ADMA/DDAH pathway is a novel, modifiable adipose tissue regulatory mechanism for nitric oxide bioavailability.

CHAPTER 5: DISCUSSION

Obesity is the most prevalent nutritional disorder in industrialised countries and is a growing problem in the developing world (Kushner, 2002). Its consequential pathologies such as insulin resistance and endothelial dysfunction are closely associated with the development of coronary heart disease and type II diabetes (Yudkin et al, 1999). The exact role of, and mechanisms by which, increased obesity promotes cardiovascular disease is poorly understood, and the extent to which these diseases are reversible with weight loss is not yet clear. Factors derived from adipose tissue may causally underlie these relationships (Recasens et al, 2004)

Obesity is affected through increases in fat cell number, cell size and function. Obesity often leads to an increase in circulating cardiovascular risk factors, which may be involved in the development of cardiovascular disease (Levenson, 2002; Sorisky, 2002). Current evidence suggests that the association between obesity and cardiovascular disease can be explained, at least in part, by novel signalling molecules, adipokines, emanating from, or expressed in, adipose tissue. While the adipose tissue is the ultimate depot for the storage of energy in the form of triacylglycerol, adipocytes also play a dynamic role, influencing processes related to energy balance through the expression and secretion of molecules which regulate energy intake and expenditure by the organism (Spiegelman and Flier 2001).

Productions of most of the adipose tissue secreted factors are likely to be affected by obesity. Since obesity is associated with multiple metabolic disorders and increased risk of cardiovascular diseases, the idea has emerged that WAT could be instrumental in this complication, by virtue of its secreted factors. There is now compelling evidence that adipocytes act as

autocrine, endocrine and paracrine cells. A wide range of signals emanates from this tissue such as leptin, tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and their soluble receptors. These molecules are expressed in and released by adipocytes and their levels increase with increasing fat mass (Lyon et al, 2003). Signals derived from this tissue going to the brain (as a central effect) as well as peripheral tissue (liver, skeletal muscle, vasculature, and adipose tissue itself). For instance, Circulating IL-6 stimulates the hypothalamic-pituitary-adrenal (HPA) axis as well as its effect on liver and induction of hepatic acute phase response.

5.1 IL-6

IL-6 plays a key role in several mechanisms that contribute to the development of CHD (Yudkin et al, 2000). IL-6 is a powerful inducer of the hepatic acute phase response and several acute phase proteins, such as CRP and fibrinogen, are potent cardiovascular risk factors. Elevated concentrations of CRP are found in patients with acute coronary syndromes, and predict future risk in healthy subjects. Elevated levels of fibrinogen, with autocrine and paracrine activation of monocytes by IL-6 in the vessel wall contribute to the deposition of fibrin. The acute phase response is associated with increased blood viscosity, platelet number and activity. IL-6 decreases lipoprotein lipase (LPL) activity and LPL levels in plasma, which increases macrophage uptake of lipids (Yudkin et al, 2000).

Stimulation of hypothalamic-pituitary-adrenal (HPA) axis by IL-6 could be associated with central obesity, hypertension and insulin resistance. Thus IL-6 is involved in the pathogenesis of CHD through a combination of autocrine, paracrine and endocrine mechanisms. IL-6 stimulates both thermogenesis

and satiety, through a range of central effects, including prostaglandin synthesis and corticotrophin releasing hormone (CRH) release (Mastorakos et al, 1993).

Circulating IL-6 concentrations increase with age and obesity, both conditions often associated with increases in adipose tissue mass (Goodpaster, 2002).

5.1.1 Pathways of IL-6 secretion

The signalling pathways involved in adipocyte secretory and its metabolic functions, which lead to obesity and its associated pathologies, are poorly defined.

The expression and secretion of IL-6 appears to be regulated by several factors. A more complete understanding of the molecular and biochemical pathways regulating the biosynthesis of this molecule and its precise mechanism of action is likely to lead to new approaches for managing not only obesity but also cardiovascular disease.

Macrophages, endothelial cells, smooth and skeletal muscle and adipose tissue all produce interleukin-6 (IL-6), but differ in the regulation of this production. For example, skeletal muscle produces physiologically significant quantities of IL-6 in response to exercise. Both post-exercise and during infection the magnitude of the cytokine response is far greater but of a shorter duration, lasting hours to days. Obesity (excess adipose tissue), on the other hand, is associated with chronic low-level elevation of circulating IL-6, largely from adipose tissue. These levels have been shown to correlate with risk factors for type 2 diabetes and coronary heart disease. A proportion of adipose secretion of IL-6 is constitutive.

Systemic IL-6 is elevated in both murine and human obesity.

Data from this work showed that basal IL-6 secretion in adipocytes and in adipose tissue was mediated through the COX-2 pathway metabolites, specifically PGE2 (chapter 2). It has been shown that in sub-cutaneous and intra-abdominal adipose tissue explants there was constitutive COX-2 expression that increased over 48h incubation, (peak at 24h). Basal IL-6 secretion mimicked the COX-2 expression and correlated with endogenous PGE2 production. Furthermore, COX inhibition significantly reduced IL-6 production from adipose tissue, as well as IL-1β-induced IL-6 secretion in differentiated 3T3-L1 adipocytes.

Exogenous PGE2 and EP agonists induce IL-6 via EP4 receptors (present in AT- SV & macrophages) and elevation in intracellular cAMP, but independent of any changes in intracellular Ca^{2+}. Understanding the specific pathway of adipose IL-6 release would allow targeted modulation of this function.

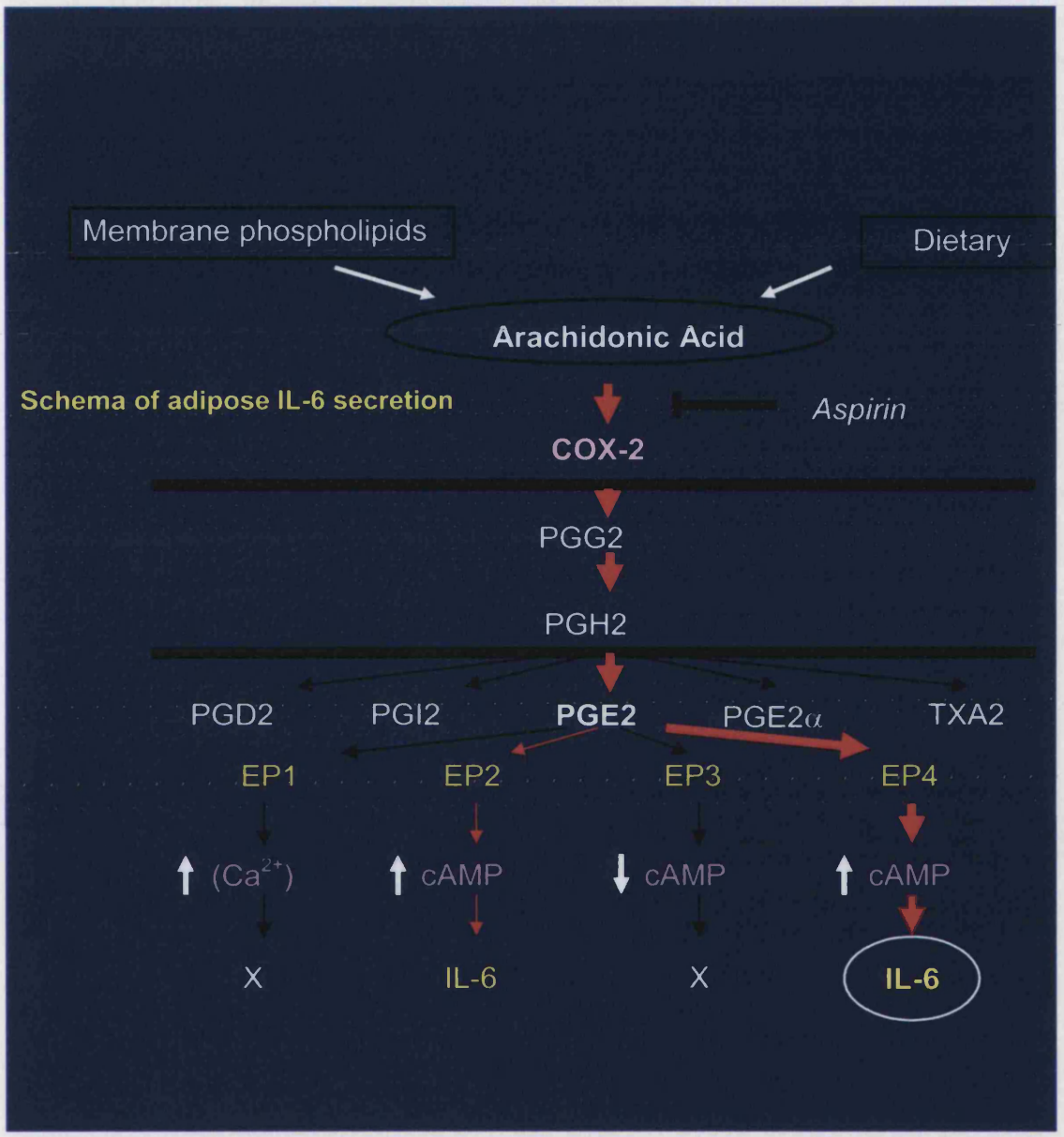


Figure 5.1: Schematic pathway of IL-6 secretion in adipose tissue and adipocytes

Basal IL-6 secretion occurs through increased COX-2 mediated PGE2 release. Aspirin, a non-selective COX inhibitor, inhibits adipose IL-6 release. Prostaglandin E signals via EP receptors (EPs). EP-1 activation signals through a rise in intracellular $[Ca^{2+}]$, However, EP-2/4 receptors raise, and EP3 decreases intracellular cAMP. PGE_2 significantly stimulates IL-6 synthesis through both EP2 and EP4 receptors and elevated intracellular cAMP. Abbreviations: COX, Cyclooxygenase; EP, PGE2 receptor; IL-6, Interleukin 6; PG, Prostaglandin.

5.1.2 IL-6 and adipogenesis

Since obesity is a condition with increased adipose tissue mass and accompanying chronic elevation in circulating levels of IL-6, the effect of IL-6 as a growth factor for adipocytes and its consequence on adipogenesis was investigated.

The effect of IL-6 on adipocytes appears to be indirect, through increased secretion of leptin and lipid accumulation without any effect on the expression of the *ob* gene. However, adipogenesis was dose dependently inhibited by the non-selective COX inhibitor, aspirin (chapter 3). This effect of aspirin on adipocyte differentiation was mediated through the COX pathway. Since IL-6 had no effect on adipogensis, the inhibitory effect of aspirin in treated adipocytes was independent of IL-6 levels. The effect of aspirin on adipogenesis was mediated through inhibition of transcription factors (PPARγ and CEBPα). Using selective COX inhibitors it was found that the adipogenic effect is mediated through COX-1, which is different from the regulation of secretion, which appears to be a COX-2 mediated effect. There is not enough *in vivo* evidence to support the effect of aspirin on weight reduction. Almost all data in the literature reflect the effect of aspirin on weight loss in subjects with normal BMI. More clinical studies are necessary to discover the effect of aspirin on BMI and specifically fat distribution in obese individuals.

5.2 ADMA/DDAH system

5.2.1 Nitric Oxide

Nitric oxide (NO) may play a key role in the control of metabolic and cardiovascular homeostasis. In recent years human and rodent adipose tissue has emerged as a potential site of NO production. NO is a messenger for a

wide variety of physiological functions. NO derived nitrite and nitrate concentrations correlate with body fat composition in humans. NO is also involved in adipose tissue biology by influencing adipogenesis, insulin-stimulated glucose uptake and lipolysis. NO induces lipid accumulation and lipogenic enzymes in rat white preadipocytes (Gaudiot et al, 1998). *In vivo*, insulin-stimulated glucose uptake in rat white adipose tissue was dependent on intact NO synthesis (Roy et al, 1998). NO inhibited lipolysis in human and rat subcutaneous adipose tissue depots (Andersson et al, 1999;Jordan et al, 2001; Klatt et al, 2000). Based on these findings, NO appears to be an important mediator of adipocyte physiology with lipogenic properties (Engeli et al, 2004).

As the principal endogenous inhibitor of NO synthase, ADMA regulates rates of NO formation.

5.2.2 ADMA

ADMA is a novel risk factor for cardiovascular disease, which inhibits NO synthesis and causes endothelial dysfunction. ADMA, by blocking NO generation, initiates or encourages atherogenesis, plaque progression and plaque rupture. A causal relationship between increased ADMA levels and endothelial vasodilator dysfunction has been shown in many of conditions related to cardiovascular disease. ADMA is primarily cleared by catabolism through the activity of dimethylarginine dimethylaminohydrolase (DDAH). The ADMA/DDAH axis in adipose tissue was investigated to explain the association between insulin resistance and endothelial dysfunction in obesity (chapter 4).

This study, for the first time, assessed the expression of DDAH and the production of ADMA from adipocytes and adipose tissue.

Both isoforms of DDAH were expressed in, and ADMA was released by, adipocytes. In organ cultures a correlation was observed between the adipose tissue mass and its release of ADMA and haploinsufficiency of DDAH 1 led to accumulation of ADMA. The release of ADMA from adipose tissue was confirmed in human subjects *in vivo,* and obese animals and humans have elevated circulating concentrations of ADMA and reduced adipose DDAH expression. Importantly, weight loss increased tissue DDAH expression and reduced ADMA. Treatment with an insulin sensitising agent, rosiglitazone, increased DDAH expression and decreased the output of ADMA from adipose tissue. The level of DDAH expression in adipose tissue is a key determinant of local ADMA levels and a target for novel therapies for obesity-associated pathologies.

Linking adipokines with ADMA/DDAH pathway will open another important regulatory pathway within the adipose tissue, as well as providing a causal link between insulin resistance and endothelial dysfunction in obesity. These results provide a novel, modifiable pathway in the tissue and a possible target for therapeutic intervention.

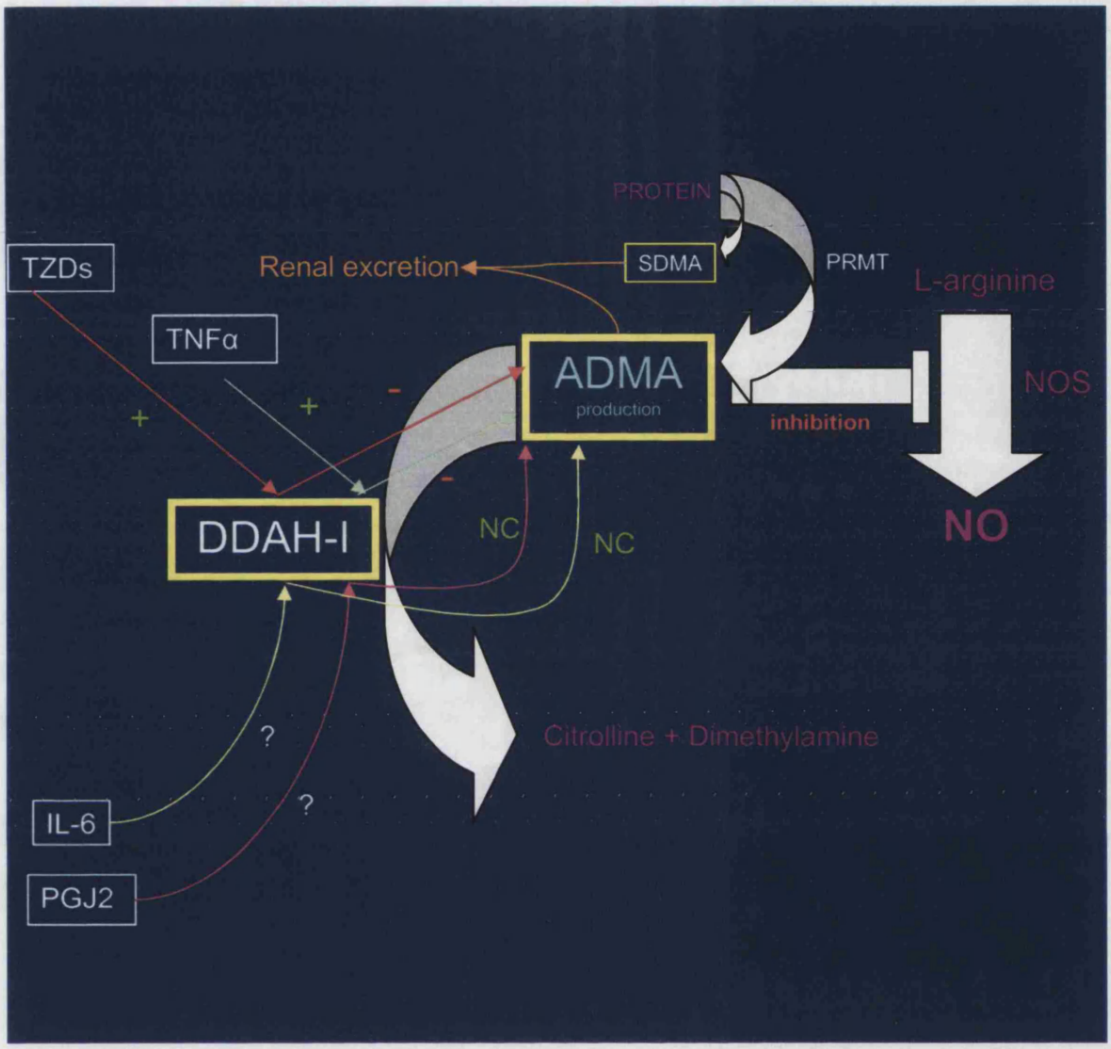


Figure 5.2: Schematic pathway of DDAH/ADMA Pathway in adipose tissue

The ADMA/DDAH pathway is a novel, modifiable adipose tissue regulatory mechanism for nitric oxide bioavailability. Adipose tissue is a source of ADMA, an endogenous inhibitor of NOS. ADMA is mainly catabolised by DDAH. Treatment of adipose tissue explants with rosiglitazone (20μM) and TNFα (10μM) increased the expression of DDAH1. Furthermore ADMA release by the tissue was reduced in TZD and TNFα treated compared to untreated tissue. The effect of IL-6 and PGJ2 on adipose tissue DDAH expression and ADMA release was not significantly different from untreated tissue. Abbreviation: ADMA, Asymmetric Dimethyl Arginine; DDAH, Dimethylarginine dimethylaminohydrolase; IL-6, Interleukin 6; NO, nitric Oxide; NOS, nitric Oxide synthase; PGJ2, Prostaglandin J2; PRMT, Protein Arginine Methyl Transferase; TNFα, Tumor Necrosis Factor α; NC, no change.

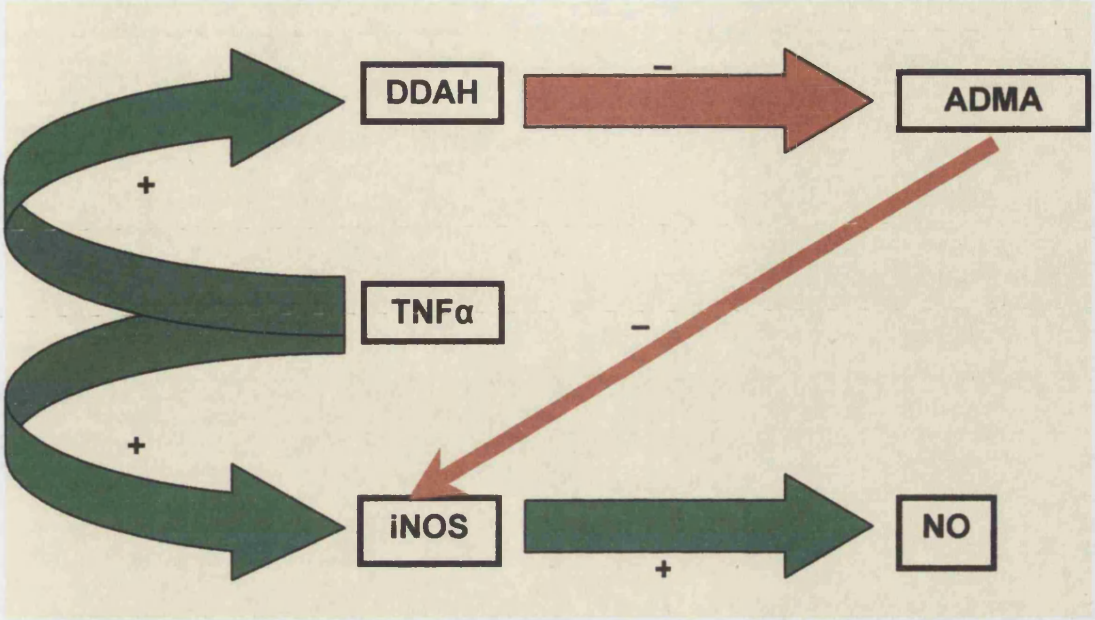


Figure 5.3: The effect of TNFα on NO production through DDAH and iNOS expression

TNFα induces DDAH-I protein expression in subcutaneous adipose tissue. Previous studies showed TNFα induces iNOS in endothelial cells. Induction of iNOS increases NO production; however increasing DDAH expression decreases ADMA levels. So it has been postulated that TNFα has the same effect on NO production in adipose tissue.

Abbreviation: ADMA, Asymmetric Dimethyl Arginine; DDAH, Dimethylarginine dimethylaminohydrolase; iNOS, Inducible nitric oxide synthase; NO, nitric Oxide; NOS, nitric Oxide synthase; TNFα, Tumor Necrosis Factor α.

5.3 Future Work

This project introduces a new area of research with many questions remain unanswered and many interventions remain to be explored. Progress in molecular biology allows us to gain more insights into cardiovascular risk factors and also face a variety of interacting pathways and increasing number of different pathomechanisms reported for individual cases. Although a great work on secretory function of adipocyte and adipose tissue has been done, there is still much left to do. Listed below are several areas that look especially attractive for future exploration.

It could be investigated:

- Pharmacologic interventions in down stream part of COX pathway of IL-6 secretion by using selective EP2 and EP4 antagonist in vitro and in vivo.
- The effect of aspirin, selective COX-1 and COX-2 inhibitors on circulating IL-6 levels in lean and obese individuals.
- The effect of aspirin and selective COX-1 and COX-2 inhibitors on the level of adiponectin and other signaling molecules from adipose tissue in vivo and in vitro.
- The effect of aspirin and selective COX-1 and COX-2 inhibitors on adipogenesis specifically in individuals with BMI more than 30 and also its effect on fat distribution
- The effect of ADMA and SDMA on transcription factors and adipogenesis
- Interventional studies aimed at lowering ADMA production from adipocyte and adipose tissue.

- The effect of dietary and pharmacological interventions with improved insulin sensitivity and endothelial dysfunction on adipose tissue expression of DDAH and release of ADMA
- Dietary and pharmacological interventions with their direct effect on adipose ADMA production and DDAH expression which could be a target for future therapies for obesity associated pathologies
- The effect of aspirin on ADMA production and also DDAH-1 and DDAH-2 expression in subcutaneous and epididymal adipose tissue with different time course and concentration
- The effect of leptin and other signalling molecules from adipose tissue on ADMA production and DDAH expression

These areas are a few of the many areas where further work might be focussed.

In the process of introducing the association between adipose tissue and the novel risk factors of coronary heart disease and type 2 diabetes it has been attempted to explore the molecular mechanism of the production of these molecules from adipose tissue and filling in as many details as was possible during a first exploration. It is our hope that other investigators will find some of these problems worthy of their attention.

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